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TITLE:
Generation of “LYmph Node Derived Antibody Libraries” (LYNDAL) for selecting fully human antibody fragments with therapeutic potential

ABSTRACT:
The development of efficient strategies for generating fully human monoclonal antibodies with unique functional properties that are exploitable for tailored therapeutic interventions remains a major challenge in the antibody technology field. Here, we present a methodology for recovering such antibodies from antigen-encountered human B cell repertoires. As the source for variable antibody genes, we cloned immunoglobulin G (IgG)-derived B cell repertoires from lymph nodes of 20 individuals undergoing surgery for head and neck cancer. Sequence analysis of unselected “LYmph Node Derived Antibody Libraries” (LYNDAL) revealed a naturally occurring distribution pattern of rearranged antibody sequences, representing all known variable gene families and most functional germline sequences. To demonstrate the feasibility for selecting antibodies with therapeutic potential from these repertoires, seven LYNDAL from donors with high serum titers against herpes simplex virus (HSV) were panned on recombinant glycoprotein B of HSV-1. Screening for specific binders delivered 34 single-chain variable fragments (scFvs) with unique sequences. Sequence analysis revealed extensive somatic hypermutation of enriched clones as a result of affinity maturation. Binding of scFvs to common glycoprotein B variants from HSV-1 and HSV-2 strains was highly specific, and the majority of analyzed antibody fragments bound to the target antigen with nanomolar affinity. From eight scFvs with HSV-neutralizing capacity in vitro, the most potent antibody neutralized 50% HSV-2 at 4.5 nM as a dimeric (scFv)(2). We anticipate our approach to be useful for recovering fully human antibodies with therapeutic potential.

Introduction:
Monoclonal antibodies (mAbs) have become an integral part of modern treatment concepts for cancer, inflammation, immunological disorders, and infectious diseases. Fully human mAbs, carrying no xenogenic components and displaying most preferable pharmacokinetic profiles, have emerged as the fastest-growing group of therapeutic antibodies entering clinical trials.1,2 Since the first report of the production of a mouse mAb through employment of B cell hybridoma fusion techniques,3 several powerful technologies for the generation of entirely human antibodies have been developed.4,5 These include non-combinatorial methodologies for the efficient immortalization of human B cells,6,7 the generation of stable human hybridomas,8 and expression-cloning of physiological variable (V) gene pairings from blood-derived human B cells of infected individuals.9 The vast majority of clinically-investigated fully human mAbs, however, are produced by either immunization of transgenic mice that are equipped with the human antibody gene repertoire,10,11 or by preparing human antibody phage display libraries. In the latter approach, random pairs of the variable genes of the heavy and light antibody chains (VH and VL, respectively) are cloned as combinatorial libraries involving bacteriophages for presentation of selectable antibody fragments.12 Since the initial description of antibody phage display,13 this technology has evolved into the most successful in vitro selection platform for human antibodies because it is robust, inexpensive, and offers great potential for automation.14,15 The first fully human phage display-derived mAbs have recently been approved by the FDA for the treatment of rheumatic and chronic inflammatory bowel diseases (adalimumab), systemic lupus erythematosus (belimumab), and inhalational anthrax infection (raxibacumab). Other phage display library-selected mAbs are currently at advanced stages of clinical development and may reach market approval within the next few years.
Over the past 20 years, a plethora of phage-derived antibody libraries have been constructed that differ in design, origin, diversity, and method of generation. Nevertheless, depending on the gene sources employed, all antibody libraries can be grouped into naïve, immune, and (semi-) synthetic libraries. Naïve libraries were amplified from naturally rearranged V genes of primary B-cell repertoires of healthy donors,16-19 whereas immune libraries were created from V genes from infected or immunized donors against a wide range of disease-related target antigens.20-32 The design of synthetic libraries involved computational approaches and gene synthesis33-37 that were partially completed by segments from natural sources.38-42 Nowadays, almost all existing commercial libraries are based on non-immune repertoires of high complexity because selection

of high-affinity binders is expected to directly correlate with the size of the functional repertoire. To theoretically allow the selection of mAbs against a virtually unlimited number of target antigens, latest state-of-art libraries reach complexities of up to 1011–1012 clones and thus may even exceed the size of individual human B cell repertoires.17,34,37 Although these libraries are highly successful in recovering target-specific and high affinity antibodies against a wide range of different antigens, they do not usually contain specificities that are developed during the course of a humoral immune response in vivo. It is nonetheless conceivable that antibodies derived from secondary and hyperimmune responses may have advantageous functional properties and improved fine-specificities over immunologically unchallenged repertoires.43,44
To exploit this particular antibody pool for therapeutic purposes, we aimed at generating a library collection of independently combinable IgG repertoires from B cells that have previously encountered defined target antigen(s) in the course of disease. In the present study, we employed lymph-node derived B cell repertoires for cloning 20 individualized phage display libraries in the scFv antibody format. Seven libraries deriving from donors with high serum titers against herpes simplex virus (HSV) were employed for panning against the viral glycoprotein B (gB) of HSV-1 that serves as an entry receptor for viral transmission.45 Our data show that high affinity scFvs with HSV-neutralizing properties could successfully be selected by this approach.

Library construction ::: Results:
The LYNDAL concept is based on the cloning of individual lymph node IgG donor repertoires. In total, 20 donor lymph nodes were included for generation of 40 individual sublibraries (20 VH/VL-kappa and 20 VH/VL-lambda repertoires, respectively). Sizes of all individual LYNDAL are listed in Table 1. On average, each sublibrary consisted of 1.1 × 108 independent members and thus 2.2 × 108 for each donor repertoire. The total size of the final LYNDAL library collection comprising 40 sublibraries was approximately 4.4 × 109 clones.

Characterization of LYNDAL repertoires ::: Results:
Selection of specific binders from phage display libraries requires expression of correctly folded scFv fragments on the phage surface. The quality of the LYNDAL repertoire was therefore characterized on both the gene and protein level. Of 1460 sequenced clones from 20 donors, 67% contained full-length scFv inserts with a similar proportion of kappa and lambda sublibraries (71% and 65%, respectively; Table S1). To further evaluate the number of solubly expressed scFvs by E. coli, we analyzed the induced periplasmic preparations of randomly picked clones from the LYNDAL collection by dot blot and western blot analysis. Eighty-two percent of LYNDAL clones investigated by dot blot screening were expressed as soluble antibody-pIII fusion proteins (Fig. 1A) with comparable expression rates of kappa and lambda light chain sublibraries (80% and 83%, respectively; Table S2). The proportion of detectable antibody-pIII fusions correlated well with that of complete inserts as analyzed by colony PCR ($r = 0.75$). Similar results were obtained in anti-pIII western blotting. Expression of intact scFv-pIII fusion proteins was shown for 27 of 30 clones chosen at random from positive dot blot signals (Fig. 1B). Thus, in total 73% of the LYNDAL clones could be expressed as complete scFv-pIII fusion proteins. On the basis of these analyses the current LYNDAL collection can be estimated to consist of 3.1 × 109 independent clones.
The primer set used for LYNDAL cloning was designed for PCR amplification of all known functional human antibody alleles and families as represented in the antibody gene database VBASE. Sequence analysis of 280 randomly picked clones (142 VH/VL-kappa and 138 VH/VL-lambda) with verified scFv insert was performed to analyze distribution and usage of germline genes and gene families actually being amplified by this primer set. We identified 90 out of the 117 VBASE annotated functional antibody genes (Fig. 2A–C). All known 22 human V gene families (Fig. 3A) and most of the VH (45 out of 51), VL-kappa (22 out of 35), and VL-lambda (23 out of 31) functional sequences were identified (Fig. 3B). The distribution pattern of the functional sequences in LYNDAL was comparable to that of three large antibody gene databases (VBASE, VBASE2, IMGT) considered to closely represent the naturally occurring distribution (Fig. 3C). Apparent differences were found for 4 families that occurred more frequently in either LYNDAL (i.e., VH6, VK4) or in the database entries (i.e., VH2, VL5).

Antibody selection from LYNDAL ::: Results:
In a next step, we assessed whether the pool of affinity-matured antibodies within the LYNDAL collection was directly accessible for antibody selection. We chose envelope glycoprotein B of HSV type 1 (gB-1) as the target for antibody selection because of the high prevalence of HSV-1 in

the adult world population (50–85%).46,47 To exclusively use HSV-experienced B cell repertoires for screening of LYNDAL, only donor libraries from patients with confirmed serum antibody response in ELISA were included. Of 17 tested serum samples, 14 LYNDAL donors (82%) were gB-reactive with OD signals at least five times greater than those of non-immunized controls (OD 0.25; SD ± 0.04). Seven libraries (No. 1, 3–8) from donors with high IgG antibody titers against gB-1, containing a total of 7.1 × 108 clones, were combined for selection. Specific binders to gB-1 were successfully enriched (Fig. 4A). ELISA screening with monoclonal phages from round 2 and 3 revealed increases of target-specific scFv phage antibodies of 21% (20 out of 96) (Fig. 4B) and 34% (33 out of 96) (Fig. 4C), respectively. Subsequent fingerprint analyses of clones with verified scFv genes (Fig. S1) resulted in the identification of 34 individual binders from 192 screened colonies, i.e., every 6th screened colony (17.7%) encoded for a gB-specific antibody with unique gene sequence.

Sequence analysis of HSV-specific LYNDAL antibodies ::: Results:
Sequence analysis revealed that the variable genes of selected scFvs derived from various germline sequences (18 out of 117) and antibody families (9 out of 22) with a predominant use of VH1 and VH3 gene families and lambda light chains (Fig. 5). By analyzing respective VH/VL pairings, 22 out of 34 scFvs were partially clonally related. To further analyze antigen-driven affinity maturation of enriched scFvs, the number of somatic hypermutations was determined on both the nucleotide (Fig. 6A) and the amino acid level (Fig. 6B). On average, the VH domain possessed more amino acid exchange mutations than the VL domain (17.4 vs. 8.6). Considering the number of nucleotide mutations (VH: 32.5, VL: 14.8), the frequency for non-silent mutations was comparable for both variable genes (VH: 54%, VL: 58%). When excluding the CDR3 and FR4 regions for analysis, the exchange mutations predominantly accumulated within the CDRs with the highest mutation frequency for CDRH2 and CDRH1, followed by CDRL2 and CDRL1 (Fig. 6C).

Functional characterization of HSV-specific antibodies ::: Results:
For further analyses, scFvs were solubly expressed in the periplasm of TG1 E. coli cells, purified by immobilized metal ion affinity chromatography (IMAC) (Fig. S2), and subjected to size exclusion chromatography (SEC). The vast majority of the investigated antibody fragments eluted predominantly as scFv monomers (Fig. S3). In flow cytometry, using Vero cells either infected with HSV-1 or HSV-2, all scFvs bound specifically to membrane-associated gB of both members of the herpes virus family, while no binding was found on uninfected Vero cells (Fig. 7). Flow cytometric competition assays of four randomly selected scFvs in the presence of a 10-fold molar excess of recombinant gB-1 further confirmed antigen specificity of LYNDAL scFvs (Fig. S4). Obtained immunofluorescence signals tended to be stronger on HSV-2 infected cells probably due to the known higher genome copy number of HSV-2 compared with that of HSV-1.48 Notably, 5 of 34 scFvs showed an almost three times greater reactivity toward HSV-1 than to HSV-2 infected cells, which could indicate binding to a non-shared epitope of gB (Fig. 7).
To accurately measure equilibrium constants (KD) of scFvs for binding to the target antigen in its natural context, we first performed flow cytometric affinity measurements of 12 randomly chosen scFv monomers on HSV-1 infected cells (Table 2). Of these, 11 scFvs bound to the target antigen with KD values in the nanomolar range. Subsequent surface plasmon resonance (SPR) affinity measurements of these clones confirmed the tight binding (r = 0.90) of selected scFvs (Table 2; Fig. S5). To evaluate the scFvs for mediating therapeutically relevant antiviral activity, we next tested their ability to prevent HSV infection in vitro using a standard plaque neutralization assay. Of eight scFvs with HSV-neutralizing activity, clone 28 exhibited the highest antiviral potency and was therefore further analyzed. We have previously shown that the valency of gB-specific antibodies may have a strong effect on their HSV-neutralizing capacity.48 Clone 28 eluted in gel filtration chromatography on a calibrated Superdex 75 column in two peaks at retention times correlating to the size of a monomer (~34 kDa, 84%) and a non-covalently associated dimeric (scFv)2 fragment (~57 kDa, 16%). Both antibody fractions were therefore separated by preparative size exclusion chromatography (Fig. S6) and further independently characterized.
Bivalent binding of the dimeric (scFv)2 28 resulted in increased avidity to glycoprotein B on the surface of HSV infected cells with apparent equilibrium constants of 7.3 nM for HSV-1 and 6.8 nM for HSV-2. The monomeric scFv 28 showed a 2-fold weak binding (KD 15.5 nM for HSV-1 and 14.8 nM for HSV-2, respectively, Fig. 8). Similar affinities of scFv 28 and (scFv)2 28 for binding to both HSV-1 and HSV-2 infected cells indicated that this antibody must recognize an epitope that is shared by both strains. Evaluation of HSV-1 and HSV-2 neutralizing activity showed that the dimeric (scFv)2 28 indeed neutralized both HSV serotypes significantly better than the monomeric

scFv (Fig. 9). Surprisingly, both monomeric scFv and dimeric (scFv)2 neutralized HSV-2 with a 14-fold and 7-fold higher efficacy than HSV-1. The efficacy of the dimeric (scFv)2 28 for neutralizing HSV-2 was even more favorable than that of a humanized mAb currently being developed for clinical applications in acyclovir-resistant disease.49

Discussion:
Humoral immune responses play a critical role in acquiring immunity against infectious disease. As demonstrated by single B cell expression cloning, single antibodies developed in the natural course of infection in patients may exhibit strong capacity for broadly neutralizing viral pathogens such as HIV-150-52 or influenza virus.53 Despite the still limited understanding of how these antibodies contribute to the actual control of especially chronic infectious diseases in a living organism, it seems clear that they undergo affinity maturation in lymphoid tissue.51 Retrieval of such particular V gene repertoires as a source for library construction is highly attractive because these repertoires may enable discovery and development of antibodies with potentially unique functional properties.
Access to human immune repertoires is generally restricted to peripheral blood and bone marrow. Here, we described an alternative approach for recovering functional mAbs from combinatorial antibody phage display libraries derived from lymph nodes as a source of immune repertoires. The underlying idea of the LYNDAL concept is based on the assumption that human immune libraries cloned from antigen-encountered antibody repertoires may have unique antigen-specific biological properties that can be exploited for therapeutic interventions.
Lymph nodes appear to be a highly valuable source for immune library cloning54 because fundamental processes of the humoral immunity take place within their germinal centers, such as somatic hypermutation, class switch recombination, and differentiation of clonally expanded B-cells into memory B and plasma cells.55,56 Moreover, lymph nodes contain a high number of antigen-encountered and activated B lymphocytes,57,58 including a conceptually interesting subset of re-entered and re-activated IgG positive memory cells harboring extensive somatic hypermutation.59 Since the selection of HIV-neutralizing mAbs from antigen-specific memory B cells has been reported,51 the memory B cell population in general might represent a crucial antibody gene pool for selecting antiviral antibodies with unique biological properties. Based on these considerations, we employed lymph node-derived (memory) B cell populations from 20 donors for cloning 20 independently combinable IgG repertoires. With a combined size of 3.2 × 109 independent antibody clones, LYNDAL represents, to our knowledge, one of the largest lymph node-derived antibody libraries to date.
To demonstrate the proof-of-concept for extracting high affinity antiviral antibodies from the combinatorial repertories of immunized donors, we combined sublibraries from individuals with high target-specific IgG titers for panning. HSV was chosen as the target for antibody selection because of the high infection prevalence in the population and the previously reported successful antibody selection from HSV-seropositive bone marrow libraries.60 Here, we have shown that screening of LYNDAL from donors with humoral response to HSV resulted in a high rate of unique high affinity binders with a broad representation of different germline sequences from various antibody families. Notably, variable regions of isolated scFvs had accumulated a particularly high degree of somatic hypermutations. As a result, the average number of mutated amino acids was higher than that reported for hyperimmunized vaccinated individuals (26.0 vs. 20.5) hypothesized to be representative for a fully matured humoral immune response.61 Thus, the LYNDAL concept allowed the selection of HSV-specific antibodies being matured during the course of a natural occurring immune response. To assess whether the high degree of somatic hypermutations would also translate into favorable functional properties of the selected antibodies, we performed HSV neutralization assays. Despite the quite limited number of analyzed clones, several scFvs exhibited potent antiviral activity. Most notably, neutralization properties of one clone (#28) were so remarkable that further exploration of the antiviral efficacy in HSV infected mice is warranted. We conclude that antigen-encountered lymph node B cell repertoires from several donors may provide a valuable alternative for selecting high affinity antibodies with immunologically unique functional properties. It remains to be shown in the future if this may also translate into antibodies with superior therapeutic activity when compared to antibodies obtained from very large synthetic or semi-synthetic libraries. By showing the value of lymph node derived-immune libraries for isolating in vivo matured mAbs with promising virus-neutralizing properties, we expect the LYNDAL concept to be also extendable to other disease-specific targets.

Ethics statement and lymph node collection ::: Materials and Methods:

The study was approved by the ethics committee of the Faculty of Medicine, Heidelberg University. All patients provided written informed consent. Lymph nodes were obtained from 20 donors undergoing surgery. Dissected lymph nodes were transferred directly into RNAlater reagent (Qiagen, 76163) and stored at −20 °C. Individual lymph nodes were thawed to room temperature (RT) and homogenized using a TissueRuptor (Qiagen). Total RNA was isolated using the RNeasy Lipid Tissue Midi Kit (Qiagen, 75842), and single stranded cDNA was prepared using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1612).

Construction of LYNDAL ::: Materials and Methods:
Libraries were generated by randomly combining amplified human VH and VL genes into phagemid vector pHENIS, a derivative of pHEN,62 containing a hexahistidine and a c-myc-tag encoding sequence. Variable domain genes were cloned as scFv fragments in VH/VL orientation linked by a 15 amino acid encoding peptide (G4S)2GGSAQ. IgG V region repertoires of human donors were amplified in a two-step, semi-nested PCR strategy using primers with a maximum of two degenerated nucleotide positions. Forward primer design was based on all functional variable gene sequences as represented in VBASE (http://www2.mrc-lmb.cam.ac.uk/vbase/). Reverse primers bound to constant genes (kappa, lambda, and CH1) or to J encoding segments and were designed on the basis of sequence information of the Kabat63 and VBASE database, respectively. VH, VL-kappa, and VL-lambda encoding gene repertoires were separately amplified from prepared cDNA in several independent PCRs using 2 units/reaction of Pfu polymerase (Thermo Fisher Scientific, EP0502). Antibody genes were PCR-amplified during the first set of PCRs (1st PCRs, 30 cycles), followed by re-amplification of PCR products (2nd PCRs, 15 cycles) to introduce cloning restriction sites by using the same PCR program: 94 °C for 3 min for initial denaturation, followed by cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min final elongation. Amplified VH gene repertoires were subcloned as SfiI/XhoI-fragments, followed by electroporation of phagemids into electrocompetent TG1 E. coli cells (Agilent Technologies, 200123). Light chain kappa or lambda repertoires were separately cloned as ApaLI/NotI-fragments into the VH-containing phagemid. Library-containing phagemid vectors were transformed by electroporation (n = 88–132 per donor) into E. coli, thus creating two independent libraries (VH/VL-kappa and VH/VL-lambda). Transformed bacteria were spread on 145 mm 2xYT agar plates containing 2% (w/v) glucose, and 100 μg/ml ampicillin (2xYT-GA). After overnight (o/n) growth at 30 °C, individual donor libraries were stored as glycerol (15% final) stocks in aliquots at −80 °C.

Characterization of LYNDAL ::: Materials and Methods:
To determine LYNDAL sizes, two transformed samples per sublibrary were randomly chosen, incubated for 1 h at 37 °C before 10-fold serial dilutions of cells were plated on 2xYT-GA agar plates. The next day, the respective library size was calculated by extrapolating the average bacterial number from dilutions with countable colonies. Up to 40 randomly picked transformants per sublibrary were further analyzed by colony PCR for successful integration of both heavy and light chain genes (fragment size ~1kb). Sequences from at least five intact, randomly chosen scFvs per sublibrary were determined and analyzed by sequence alignment software DNAPLOT (http://www2.mrc-lmb.cam.ac.uk/vbase/dnaplot2.php) to identify the closest germline V, (D), and J segment genes as deposited in VBASE. Complementarity-determining regions (CDRs) were additionally analyzed by using the Fab Analysis tool (http://www.vbase2.org/vbscAb.php) by aligning the scFv sequences to the VBASE2 database.64 Relative gene family occurrences of sequenced LYNDAL clones were calculated and compared with the mean distribution of all functional V gene entries of human antibody databases VBASE, VBASE2, and IMGT-GENE.65 Clones with non-functional reading frames, orphans, and pseudogenes, were excluded from the analysis.
Each sublibrary was analyzed for soluble expression of antibody-pIII fusions in E. coli by dot blot and western blot analysis. Individual colonies were inoculated in 96-well plates in 2xYT-GA medium and grown o/n at 37 °C on a shaker. Induction plates containing 150 μl 2xYT-A with 0.1% glucose were inoculated with 3 μl o/n cultures and incubated for an additional 3 h until soluble expression of antibody-pIII fusions was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After o/n expression at 28 °C, pelleted bacteria (3000 xg, 10 min, 4 °C) were resuspended in periplasmic preparation buffer (30 mM TRIS-HCl, pH 8.0, 1 mM EDTA, 20% sucrose) containing 50 μg/ml freshly prepared lysozyme and incubated for 30 min on ice. Then $MgSO_4$ was added to a final concentration of 10 mM. Clarified periplasmic fractions (3000 xg, 20 min, 4 °C) were either applied onto nitrocellulose membranes using a Minifold I Dot Blot

system (Whatman) for dot blot analysis (100 µl/dot) or onto 12% SDS-PAGE under reducing conditions for western blot analysis. Antibody-pIII fusions were detected using either peroxidase-conjugated 9E10 anti-c-myc antibody (1 µg/ml; Roche Diagnostics, 11814150001) or mouse anti-pIII antibody (0.5 µg/ml; MoBiTec, PSKAN3) as first and goat anti-mouse IgG peroxidase conjugate (0.02 µg/ml; Jackson ImmunoResearch Laboratories, 115-035-008) as secondary antibody. Peroxidase activity was detected by chemiluminescence using ECL substrate (Thermo Fisher Scientific, 32106) and chemical film Curix HT1.000G Plus (Agfa HealthCare).

Antibody selection ::: Materials and Methods:
Selection of herpes simplex virus specific scFvs, using antigen-coated immunotubes, was performed as described previously66 with the following modifications. Antibody repertoires of seven LYNDAL donors were separately packaged by inoculating 2× YT-GA medium (200 ml/donor) with corresponding library glycerol stocks, followed by superinfection with helper phage VCSM13 (Agilent Technologies) at a multiplicity of infection of 15. Expression of antibody-pIII fusions was induced by resuspending the pelleted bacteria in induction medium (2× YT, 100 µg/ml ampicillin, 25 µg/ml kanamycin, 0.5 mM IPTG) and incubation of combined libraries on a shaker at 28 °C for 6 h. Phages were purified during two PEG-precipitation steps by incubation o/n at 4 °C and 1 h on ice, respectively. After pre-incubation of 2 × 1013 t.u. (transducing units) in 2% MPBS for 1 h at RT, phages were applied without depletion step to a gB(724tHis)-coated67 (20 µg/ml in PBS) and blocked immunotube. Binding of phages to target protein gB-1 occurred during roll-over shaking for 90 min and 30 min rest at RT. After washing (5× PBST and 5× PBS), bound phage antibodies were eluted with acid solution (0.1 M glycine-HCl, 0.5 M NaCl, pH 2.2) by incubation for 8 min at RT under rotation. Eluted phage antibodies were neutralized (pH 7.4) with 1 M TRIS-HCl pH 9.5 and added to log-phase TG1. To rescue non-eluted phages, the selection tube was additionally filled with log-phase bacteria. After infection at 37 °C, both cultures were combined before continuing as described.66 Two further selection rounds were performed by preparing 160 ml start cultures and increasing the stringency during panning by employing less gB-1 antigen for coating (5 µg/ml) and more washing cycles (10× for round 2 and 15x for round 3 with PBS and PBST, respectively).

Phage ELISA ::: Materials and Methods:
Polyclonal phage ELISA (ppELISA) was performed as described previously66 by adding 1011 t.u./well of rescued phages to the antigen-coated microtiter plates. For monoclonal phage ELISA (mpELISA), single colonies from positively enriched panning rounds were inoculated into 96-well microtiter plates containing 100 µl of 2× YT-GA. Cultures were shaken o/n at 37 °C and used for inoculation of an infection plate with fresh medium; the master clones were stored by adding glycerol. After incubation of infection plates for 2 h at 37 °C, bacteria were superinfected with 108 t.u./well of helper phages at 37 °C, followed by re-suspension of the pellets in induction medium for antibody-pIII expression o/n at 28 °C under shaking. The next day, each clone was analyzed for binding to coated selection antigen (1 µg/ml gB-1 in PBS) and control antigen (2% MPBS), respectively, by incubation of blocked wells (2% MPBS for 1 h at RT) with 100 µl/well phage supernatant in 2% MPBS for 1 h at RT. Detection using an HRP-conjugated anti-M13 antibody (GE Healthcare, 27-9421-01) was performed as described.66 The absorbance was read at 450 nm using a GENios Plus reader (Tecan). Readings for gB-1 ten times higher than the average signal for milk protein were considered to be antigen-specific binding.

Characterization and expression of enriched scFvs ::: Materials and Methods:
The integrity of the scFv gene from specific binders was assessed by colony PCR and re-amplified genes were digested with BstNI for fingerprint analysis. After sequencing, germline sequences and respective antibody gene families were determined by DNAPLOT, followed by the construction of phylogenetic trees with the Phylogeny.fr web tool.68 Somatic hypermutation analysis of selected scFvs was performed by employing the IMGT/V-QUEST online tool.69
ScFv encoding genes were PCR-amplified and subcloned as SfiI/NotI fragments into expression vector pAB170 for soluble protein expression in the bacterial periplasm (o/n at 18 °C) as described previously.71 Hexahistidine-tagged scFvs were purified by IMAC with Ni-Sepharose 6 Fast Flow (GE Healthcare, 17-5318-01) according to the manufacturer's recommendations. The oligomeric state of IMAC-purified scFvs was analyzed after buffer exchange to PBS by SEC on a Superdex 75 10/300 GL column using an ÄKTA FPLC system (both GE Healthcare). The column was calibrated using gel filtration low molecular weight standards (Amersham Biosciences, 17-0442-01). Elution profiles were recorded by monitoring the absorbance at 280 nm and elution

volumes determined with the Unicorn software 5.11. If necessary, scFvs were further purified to homogeneity by preparative SEC on a HiLoad 16/60 Superdex 75 column (GE Healthcare). Purified scFvs were concentrated with centrifugal filter units, filter sterilized, and concentrations were measured spectrophotometrically.

Target binding analysis on virus infected cells ::: Materials and Methods:
Specific binding of purified scFvs to native gB protein was evaluated by flow cytometry using the African green monkey kidney cell line Vero (ECACC, 84113001) either infected or non-infected with HSV-1(F) or HSV-2(G). Infection and fluorescence measurements were performed as described previously48 by detection of bound scFvs (100 nM) with mouse anti-c-myc IgG (5 µg/ml) and goat anti-mouse fluorescein isothiocyanate (FITC) conjugate (7.5 µg/ml, Jackson ImmunoResearch Laboratories, 115–095–008). Fluorescence was measured on a FACSCanto II flow cytometer and mean fluorescence intensity calculated using FACS Diva software (Becton Dickinson). For determination of equilibrium-binding curves, HSV-1 or HSV-2 infected Vero cells were incubated in triplicate with scFvs dilutions (0.03–1000 nM) followed by detection as described above. Background fluorescence was subtracted from measured median fluorescence intensities, and relative affinities were calculated by nonlinear regression using GraphPad Prism version 5.0 (GraphPad Software).

SPR biosensor binding analysis ::: Materials and Methods:
Binding kinetics were determined by SPR on a BIACORE 2000 system (GE Healthcare). A standard coupling protocol was employed for immobilizing gB(724tHis) via exposed primary amines on a CM5 sensor chip (GE Healthcare, BR-1003–99). Surface activation of flow cells No.1 and No.2 for 7 min was performed with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.4 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) at a flow rate of 5 µl/min. After immobilization of the ligand (100 µg/ml in 10 mM sodium acetate, pH 4.7; 7 min, flow rate 5 µl/min) at a density of 230 RU on flow cell, 2 residual reactive sites were blocked by injection of 1 M ethanolamine, pH 8.5 for 7 min at 5 µl/min. Flow cell 1 served as a reference surface. To measure kinetics, increasing concentrations (1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, and 1000 nM) of purified monomeric scFvs were injected in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% P20, pH 7.0) at a flow rate of 10 µl/min and 25 °C. Analyte-ligand association and dissociation phases were monitored for 3 min and 30 min, respectively. Surfaces were regenerated in running buffer for further 30 min. The data were globally fitted with the Langmuir 1:1 binding model using the BIAevaluation 4.1.1 software.

Neutralization of HSV-1 and HSV-2 ::: Materials and Methods:
Neutralizing activity of LYNDAL antibodies was determined in a plaque reduction assay as described previously.49 The humanized version of a highly potent HSV-neutralizing antibody was used as positive control.49