Methods

Generation of human bispecific common light chain antibodies by combining animal immunization and yeast display

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Abstract
Bispecific antibodies (bsAbs) pave the way for novel therapeutic modes of action along with potential benefits in several clinical applications. However, their generation remains challenging due to the necessity of correct pairings of two different heavy and light chains and related manufacturability issues. We describe a generic approach for the generation of fully human IgG-like bsAbs. For this, heavy chain repertoires from immunized transgenic rats were combined with either a randomly chosen common light chain or a light chain of an existing therapeutic antibody and screened for binders against tumor-related targets CEACAM5 and CEACAM6 by yeast surface display. bsAbs with subnanomolar affinities were identified, wherein each separate binding arm mediated specific binding to the respective antigen. Altogether, the described strategy represents a combination of in vivo immunization with an in vitro selection method, which allows for the integration of existing therapeutic antibodies into a bispecific format.

Key words: antibody, bispecific antibody, common light chain, high-throughput screening, yeast surface display

Introduction
Monoclonal antibodies (mAbs) have become substantial molecules for therapeutic applications. For years, they maintained to be the bestselling class of biologics with economic growth rates up to 18% (Aggarwal, 2014). By 2014, 47 mAbs had reached clinical approval in the USA and Europe, among them 4 of the 10 best selling drugs. Furthermore, those molecules demonstrate applicability for a wide range of clinical indications, including cancer, inflammatory and infectious diseases (Nelson et al., 2010; Lo et al., 2014; Ecker et al., 2015). Potential limitations of conventional mAbs (e.g. insufficient efficacy and selectivity) can be addressed by the generation of modified antibody formats such as antibody drug conjugates or bispecific antibodies (bsAbs) (Liu, 2014). While conventional mAbs have a defined specificity for only one antigen, bsAbs are able to simultaneously bind two distinct epitopes on the same or different antigens. One prominent application example is the retargeting of immune effector cells toward tumor cells proven by two bsAbs, Catumaxomab (Removab) and Blinatumomab (Blincyto), both of which already reached market maturity (Kontermann and Brinkmann, 2015). All in all, over 30 bsAbs are currently under clinical development indicating the significant interest of the pharmaceutical industry to develop these antibody-based therapeutics (Kontermann and Brinkmann, 2015; Thakur and Lum, 2016). Although the combination of two distinct targeted epitopes opens up a broad range of novel modes of action,
manufacturability might be challenging for many bispecific formats. In this regard, it has to be distinguished between IgG- and non-IgG-like bsAbs (Fan et al., 2015). Non-IgG-like bsAbs, like bispecific T cell engager molecules, show a preferable manufacturability profile regarding production and purification due to their composition of only one peptide chain (Baueule and Reinhardt, 2009). Disadvantages of such ‘Fc-less’ formats are their tremendously reduced circulation half-lives as well as the lack of all other potentially desired Fc-driven effector functions (Kontermann and Brinkmann, 2015).

The aforementioned drawbacks can be avoided by the construction of IgG-like bsAbs which are mimicking the design of natural IgG-molecules. However, those formats have the premise that specific pairing of two distinct heavy and light chains needs to be achieved (Supplementary data Fig. S1A) (Klein et al., 2012). Several methodologies for specific heavy chain heterodimerization have been described so far, including quadrroma technology (Lindhofer et al., 1995), knobs into holes (Ridgway et al., 1996), strand-exchanged engineered domains (SEED) (Davis et al., 2010; Muda et al., 2011) and others. The SEED-technology utilizes the fact that human IgG- and IgA-heavy chains are not able to dimerize, due to different interface residues in the CH3 domains of IgG- and IgA-heavy chains. Within the two different SEED-CH3 domains, β-strand segments of IgG and IgA are asymmetrically exchanged, yielding preferentially heterodimers that are not impaired in any kind of Fc mediated effector functions (Davis et al., 2010). While for the generation of bsAbs the problem of specific heterodimerization of different heavy chains could be solved by the methods mentioned above, IgG-like bsAbs also require the correct pairing of two distinct light chains. Random light chain association would otherwise lead to a mixture of species with statistically only 25% correctly assembled antibodies (Supplementary data Fig. S1B).

One option to bypass this issue is apparently the use of a common light chain combined with two different heavy chains. BsAbs based on common chains have the advantage that no additional engineering of the antibody Fc (common heavy chain) or Fab (common light chain) moieties is required, which could have detrimental effects on stability and immunogenicity of the engineered molecules. Apart from that, only three peptide chains need to be expressed which is a clear advantage in terms of manufacturability. Especially the utilization of a common light chain seems to be a straightforward solution to avoid Fab mispairings. This approach makes use of the well-known fact that for many antibodies the main driver for affinity and specificity is the heavy chain. Thus far, common light chain antibodies were isolated from phage scFv display libraries with a restricted light chain diversity (Merchant et al., 1998) or phage Fab libraries with a unique light chain (Jackman et al., 2010). Alternatively, animals were immunized with different antigens and mAbs were isolated using the classical hybridoma technology. Then, a set of heavy and light chain combinations was generated and tested individually for retained antigen binding (Smith et al., 2015). One common light chain bsAb targeting factor IX and factor X for the treatment of hemophilia A has already progressed to clinical phase III (Shima et al., 2016).

In this study, we describe a novel approach to generate IgG-like bispecific human common light chain antibodies by combining immunization of transgenic rats with yeast surface display (YSD) antibody screening (Fig. 1; Supplementary data Fig. S2) (Boder and Wittrup, 1997). For proof of concept, rats expressing a human immunoglobulin repertoire (Ma et al., 2013) were immunized with cancer related antigens CEACAM5 and CEACAM6, both linked to tumor progression and metastasis (Tchoupa et al., 2014). Evolved heavy chain diversities were isolated from immunized rats and separately combined with a common light chain in two YSD Fab libraries. To further challenge the generalizability of this approach, repertoires were also combined with the light chain of humanized Cetuximab (huC225) representing a highly characterized molecule. After selection by fluorescence activated cell sorting (FACS), isolated variants were tested for their ability to bind to CEACAM5 and CEACAM6 followed by the construction of bsAbs utilizing the SEED-technology for heavy chain heterodimerization. We were able to identify high affinity, fully human IgG-like common light chain bispecific SEEDbodies showing simultaneous binding to both recombinant antigens as well as specific binding to cells expressing the respective antigens.

### Materials and Methods

#### Immunization of OmniRats

OmniRats (OmniAb) were immunized with vaccination vectors encoding for CEACAM5 and CEACAM6 DNA by Aldevron (Freiburg, Germany) as described previously (Perterm et al., 1995). Silencing of endogenous Ig loci was achieved by digestion with designer zinc finger nucleases, followed by error-prone intrinsic repair mechanisms. Human Ig loci encoding DNA was microinjected into fertilized oocytes in order to integrate the genes into the genome (Geurts et al., 2009; Osborn et al., 2013).

#### Plasmids

Vectors used for antibody display on yeast cells were based on the pYDI plasmid backbone (Yeast Display Vector Kit, version D, #V835-01, Thermo Fisher Scientific). The heavy chain plasmid contained a tryptophan auxotrophic marker for selection in yeast and an ampicillin resistance marker gene for selection in E. coli cells, whereas the light chain plasmid contained kanamycin resistance and
leucine marker genes. Furthermore, the heavy chain plasmid encoded for the AGA2 signal peptide, followed by the antibody VH region genetically fused to the CH1 region of IgG1, which allowed surface presentation of a VH-CH1-(G4S)-(G4S)3-Aga2p fusion protein. The light chain plasmid encoded for the mAMpp8 signal sequence (Rakestraw et al., 2009) for soluble secretion, followed by the sequence for the constant kappa region. Expression of antibody heavy and light chain genes was under control of the galactose inducible promoter (GAL1). The gene sequences encoding for the common light chain and the light chain of humanized Cetuximab (huC225) were synthesized by GeneArt (Thermo Fisher Scientific) and introduced into the light chain expression plasmid by homologous recombination. Vectors used for soluble secretion of full-length IgG-molecules, SEEDbodies as well as glycosylphosphatidylinositol (GPI) anchored antigens in mammalian cells were based on pTT5 plasmid backbone (Expresso CMV based system, Lucigen) with ampicillin resistance marker genes for selection in E. coli.

**Yeast strains and media**

The *Saccharomyces cerevisiae* strain harboring plasmids encoding for the antibody heavy chain was EBY100 (MATa URA3-52 trp1 leu2Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL1 (pU211:URA3)) (Thermo Fisher Scientific). Plasmids encoding for antibody light chains were accommodated in *S. cerevisiae* strain BJ5464 (MATa URA3-52 trp1 leu2Δ1his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL1) (American Type Culture Collection). Yeast cells were cultivated in Yeast Peptone Dextrose (YPD) medium composed of 10 g/l yeast extract (BD), 20 g/l peptone (EMD Millipore), 20 g/l dextrose (EMD Millipore) and 10 ml/l penstrep (Gibco). Transformed haploid EBY100 or BJ5464 cells were grown in liquid medium using minimal SD-base (Clontech) supplemented with commercially available dropout mix (Clontech) bearing all essential amino acids except tryptophan or leucine (Clontech), supplemented with 5.4 g/l Na2HPO4 and 8.56 g/l NaH2PO4 × H2O (EMD Millipore), respectively. Induction of gene expression was carried out in 5 g dropout medium wherein SD-base was replaced by galactose containing SG-base (Clontech). Furthermore, medium was supplemented with 11% w/v PEG8000 (Euroclone). For selection of diploid yeast cells, double drop-out-mix bearing all essential amino acids except tryptophan and leucine was used (Clontech). In addition, minimal SD Agar base supplemented with respective drop-out mixes was used for preparation of agar plates.

**RNA extraction, cDNA-synthesis and library generation**

Extraction of total RNA from 1 × 107 lymph node cells was performed with the commercially available kit RNeasy Minikit (Qiagen) according to the manufacturer’s protocol. Following RNA extraction, cDNA-synthesis was executed using random hexamer primers and 50 µl RNA extract, 20 µl RT-buffer, 40 µl 25 mM MgCl2, 20 µl 0.1 M DTT, 10 µl RNase Out and 10 µl SuperScript III reverse transcriptase (all components from SuperScript III First-Strand Kit, Thermo Fisher Scientific). The reaction was incubated for 5 min at 25°C followed by an incubation for 60 min at 50°C and a reaction stop at 85°C. Afterwards, 1 µl RNaseH was added and the solution was incubated for 20 min at 37°C. The obtained cDNA was stored at −20°C. Variable antibody regions were amplified from cDNA in two successive PCR reactions using Q5 High-Fidelity 2x Master Mix and 50 µl reaction volume (NEB) under the following conditions; PCR1: 95°C for 120 s, 30 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 90 s; PCR2: 95°C for 120 s, 30 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 45 s. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). In PCR1, 12 different reactions were prepared with 5 µl cDNA using unique forward primers annealing to germline leader sequences and one reverse primer annealing to rat CH1 domain. The second PCR reaction was performed to incorporate 5’ and 3’ overhangs complementary to acceptor plasmids. For this, 50 ng of pooled and purified product from PCR1 was amplified with degenerated primers annealing to variable heavy chain region framework1 and framework4. PCR products were then pooled, purified and subsequently used for library generation. The acceptor vector was linearized with restriction enzymes NotI and EcoRI (NEB). Heavy chain library generation in *S. cerevisiae* EBY100 cells was performed as described in detail by Benatul et al., (2010). Within each of 10 transformation reactions per library (CEACAM5 and CEACAM6), 4 µg digested plasmid as well as 6 µg PCR product were used. Library sizes were calculated from serial dilution plates with transfected cells. Yeast mating was applied to combine the generated heavy chain diversities (in EBY100; MATa) with the common light chain (in BJ5464; MATa) (Weaver-Feldhaus et al., 2004). Therefore, cells were grown in either SD-Trp or SD-Leu medium at 30°C and 200 rpm for 24 h. Next, 1 × 109 EBY100 cells and 1 × 109 BJ5464 cells were centrifuged and resuspended in 1 ml YPD medium and incubated on YPD agarplates at 30°C for 24 h. Diploid cells were then grown in SD-Trp-Leu medium and passaged three times prior to freezing to ensure growth of diploid cells. Library sizes were determined by plating of serial dilutions using double selective agar plates.

**Screening of libraries**

Transformed libraries were grown overnight in SD-Trp-Leu medium at 30°C and 200 rpm. For induction of gene expression, cells were used for inoculation of SG-Trp-Leu medium at 1 × 107 cells/ml followed by incubation at 20°C and 200 rpm for 48 h. Detection of antigen binding was performed either by using Penta-His Alexa Fluor 647 Conjugate antibody (Qiagen) or antigen-biotinylation (EZ-Link Sulfo-NHS-LC-Biotin; Thermo Fisher Scientific). All labeling steps were performed for 30 min with 1 × 107 cells per 20 µl on ice.

Preparation of cells for sorting was carried out by washing cells with PBS, with light chain specific goat F(ab')2 anti-human kappa R-phycocerythrin (R-PE) or Alexa Fluor 647 conjugate (SouthernBiotech, 1:20 diluted) for detection of surface display. Afterwards cells were incubated with antigen (CEACAM5 or CEACAM6 extracellular domain), washed with PBS and stained with Penta-His Alexa Fluor 647 Conjugate antibody (1:20 diluted). Alternatively, biotinylated antigen and Streptavidin-647 conjugate (Thermo Fisher Scientific) was used.

After washing with PBS, cells were ready for sorting. All selections were performed with MoFlo Legacy cell sorter (Beckman Coulter) and Summit 5.3 software and sorted cells were subsequently transferred to SD-Trp-Leu medium and regrown at 30°C and 200 rpm. The CEACAM5 immune library was sorted over three sequential rounds (R1, R2, R3: 1 µM CEACAM5; Supplementary data Fig. S3); whereas, the CEACAM6 immune library was sorted over two rounds (R1: 2 µM; R2: 1 µM CEACAM6; Supplementary data Fig. S3). Libraries harboring the huC225 light chain were sorted for two rounds each (1 µM).

**Cloning, expression and purification of IgG-molecules**

VH regions and the common light chain VL region were amplified from pYD vectors with primers introducing 5’ and 3’ overhangs...
complementary to target pTT5 plasmids for full-length IgG-expression (Expresso CMV based system, Lucigen). Linearized pTT5 vectors and PCR products were used for transformation of E. coli One Shot Top10 chemically competent cells (Thermo Fisher Scientific) and assembled by homologous recombination (Bubeck et al., 1993). After sequence verification of cloned constructs, Exp293 cells were transiently transfected with expression vectors following the instructions of the manufacturer (Thermo Fisher Scientific). Five days post transfection, antibody containing supernatants were harvested and purified by Antibody Purification Kit and Spin Columns with Presep-A Media (Merck KGaA).

Cloning, expression and purification of SEEDbodies
The SEED technology was applied to achieve heterodimerization of the antibody heavy chains (Davis et al., 2010). VH regions of IgG molecules that showed specific binding to their respective antigens were subcloned into pTT5 plasmids with SEED-AG-chain (pTT5-AG; CEACAM5 antibodies from common light chain screening; CEACAM5 and CEACAM6 antibodies from huC225 screening) and SEED-GA-chain (pTT5-GA; CEACAM6 antibodies from common light chain screening; huC225 antibody). Since the SEED-AG chain is known to form heterodimers only, a His tag was introduced to the 3’ end of the antibody encoding sequence on the pTT5-AG plasmid to allow a purification of heterodimeric SEEDbodies via ion metal affinity chromatography (IMAC). Variable regions of isolated antibodies were amplified with primers introducing an Ncol site 5’ and an Sall site 3’ to the variable antibody region and PCR products as well as acceptor plasmids were digested with restriction enzymes Ncol and Sall (New England Biolabs) and subsequently ligated using Quick Ligation Kit (New England Biolabs). After transformation of E. coli One Shot Top10 chemically competent cells (Thermo Fisher Scientific) with ligated plasmids, cloned constructs were verified by sequencing and used for transient transfection of Exp293 cells (Thermo Fisher Scientific). Regarding the expression of one armed SEEDbodies, antibody VH encoding pTT5-AG vectors and a pTT5 plasmid encoding for an SEED-GA-Fc only were used for transfection. After 5 days of production, supernatants were harvested and SEEDbodies were purified by IMAC with His Trap HP columns (GE Healthcare) and AKTAExpress chromatography system (GE Lifesciences). For this, a linear imidazole gradient ranging from 15 to 250 mM was applied, fractions were collected and analyzed via SDS-PAGE. SEEDbody containing fractions were pooled and the buffer was exchanged to PBS using Amicon Ultra-4 Centrifugal Filters (EMD Millipore).

Biolayer interferometry
Binding kinetic measurements were performed using the Octet RED System (ForteBio, Pall Life Science) at 30°C and 1000 rpm agitation (ForteBio, Pall Life Science). First, antibodies were immobilized on pre-wet (10 min in PBS) anti-human Fc biosensor at 5 μg/ml in PBS. Antibodies were captured on AHC sensors for 25 s followed by sensor rinsing in kinetics buffer (KB; PBS, 0.1% Tween 20 and 1% bovine serum albumin, BSA) for 180 s. For detailed kinetic analyses, association to CEACAM5, CEACAM6 and EGFR extracellular domain (varying concentrations ranging from 3.125 to 100 nM in KB) was measured for 400 s followed by dissociation for 900 s (in KB). In each experiment, one control was measured, wherein the captured antibody was incubated with KB instead of antigen. The resulting data were subtracted from all other binding curves. Evaluation of processed binding curves was performed with the ForteBio data analysis software 8.0 using a 1:1 binding model after Savitzky–Golay filtering.

One-tip measurements were used for qualitative binding analyses of IgGs. For this, association with 100 nM antigen (CEACAM5 or CEACAM6) was executed for 300 s and dissociation for 600 s. In case of the simultaneous binding experiments, SEEDbodies were captured on pre-equilibrated AHC biosensors followed by a sensor rinsing in KB (180 s) and association with CEACAM6 (100 nM in KB; 400 s; or CEACAM5 in case of huC225 antibodies) and another association in CEACAM5 (100 nM in KB; 400 s; or EGFR in case of huC225 antibodies). Controls were introduced where either CEACAM5, CEACAM6 or EGFR were replaced by kinetics buffer.

Cellular binding of SEEDbodies on Exp293, MDA-MB-231 and MKN-45 cells
Cellular binding assays were performed with 1 × 10⁷ cells per cavi on a 96-well plate. Binding of SEEDbodies was evaluated with Exp293 cells transiently transfected with CEACAM5-GPI and CEACAM6-GPI encoding pTT5 vectors, MDA-MB-231 and MKN-45 cells. Two days post transfection, Exp293 cells were harvested by centrifugation whereas MDA-MB-231 and MKN-45 cells were trypsinated from the culture flask first and then spun down. Cells were washed three times with 1% BSA (w/v) containing PBS followed by incubation with the respective SEEDbody (100 μl; 10 μg/ml SEEDbody in 1% BSA (w/v) PBS) for 1 h. Afterwards cells were washed and incubated with the fluorescently labeled secondary antibody (Alexa Fluor 488-conjugated AffiniPure goat anti-human IgG, Fcy fragment specific (Jackson Immuno Research)) for 1 h (100 μl; 10 μg/ml SEEDbody in 1% BSA (w/v) PBS), washed again and stained with propidium-iodide (200 μl; 1:200 dilution in 1% BSA (w/v) PBS (Thermo Fisher Scientific)). Cells were analyzed (5000 events) using a Guava EasyCyte HT flow cytometer device (EMD Millipore).

Ethics statement
Experimental procedures and animal care were in accordance with EU animal welfare protection laws and regulations. We confirm that all experimental protocols were approved by a licensing committee from the local government (Landesuntersuchungsamt, Koblenz, Germany).

Results
Library generation, selection and identification of hit candidates
One avenue for the generation of mAbs with high affinity and specificity is the immunization of rodents. In the current study, transgenic rats (OmniRat; OmniAb), harboring fully human variable antibody regions, were DNA-immunized with either CEACAM5 or CEACAM6. For the isolation of the naturally evolved antibody repertoires from OmniRats, lymph nodes were resected and homogenized followed by the extraction of total RNA from 1 × 10⁷ cells and subsequent cDNA-synthesis using random hexamer primers. The variable regions of antibody heavy chains were amplified in two subsequent PCR reactions. According to the separate immunization approaches against CEACAM5 and CEACAM6, two YSD libraries were constructed by homologous recombination in haploid EBY100 (mating type a, MatA) cells with sizes of ~3 × 10⁶ clones each. Library genes encoding for the heavy chain variable domains were incorporated into YSD vectors harboring an IgG1 CH1 domain and the yeast cell surface anchoring subunit Aga2p. The light chain plasmid for soluble secretion of the common
light chain (IGKV3-15*01) was generated accordingly in haploid BJ5464 cells. Yeast mating was applied to generate Fab YSD libraries by combining the heavy chain repertoires harboring EBY100 with common light chain harboring BJ5464 cells resulting in library sizes of $2 \times 10^8$ (CEACAM5) and $1 \times 10^8$ (CEACAM6) individual clones, respectively (schematically illustrated in Supplementary data Fig. S2). The two resulting libraries were screened separately by FACS against recombinant CEACAM5 and CEACAM6. Target binding was detected by indirect fluorescence staining using either biotinylated antigen and streptavidin, R-PE conjugate (CEACAM5 Round 3) or unmodified antigen (His tagged) and anti-Penta-His Alexa Fluor 647 conjugated antibody. For bivariate selections, the Fab display levels were analyzed simultaneously with light chain specific goat F(ab')2 anti-human kappa R-PE or Alexa Fluor 647 conjugate. Cell fractions associated with specific antigen binding signals were enriched throughout three (CEACAM5) and two (CEACAM6) consecutive rounds of selection. While the unselected libraries only depicted $\approx 1\%$ of double-positive (Fab display and antigen binding) events in flow cytometry, the enrichments resulted in the accumulation of $\approx 19\%$ (CEACAM6) and $\approx 39\%$ (CEACAM5) positives (Fig. 2A; screening plots in Supplementary data Fig. S3). To monitor the enrichment on a sequence level, 100 single clones from unselected and selected repertoires were isolated and analyzed by sequencing for their complementary determining region (CDR) diversities within the heavy chain. For this, a clustering strategy was applied, where each cluster contained a diverse set of sequences that showed $\approx 90\%$ identical CDR residues. The resulting number of clusters was divided by the total number of sequences used for analysis and can be considered as enrichment factor (Fig. 2B). While initial CDR diversity was calculated to be $61\%$ and $70\%$, sequence diversity of selected repertoires was reduced to $19\%$ (CEACAM5) and $13\%$ (CEACAM6). From both selection campaigns the two variants displaying the highest prevalence in the most abundant clusters were selected for further analyses. Those sequences were formatted into full-length IgG1 by subcloning into pTT5 mammalian expression vectors. In total, four variants isolated from the CEACAM5 (C5_A, C5_B) or from the CEACAM6 immune library (C6_A, C6_B) were produced in mammalian cell cultures followed by purification via protein A affinity chromatography. To evaluate affinity and specificity (both antigens share $86\%$ sequence identity) of the selected variants, biosensor interferometry (BLI) measurements were performed utilizing recombinant CEACAM5 or CEACAM6 protein. Specific antigen binding of all four selected antibody variants could be confirmed, as clones C5_A and C5_B (CEACAM5 selection) exclusively recognized CEACAM5 while clones C6_A and C6_B (CEACAM6 selection) only bound to CEACAM6 (Fig. 3). Furthermore, all variants were subjected to detailed binding kinetic analyses using BLI (Supplementary data Fig. S4). Results indicate high affinity antigen binding in the single-digit or subnanomolar range. Amino acid composition of the heavy chains CDRs can be found in Supplementary data Fig. S5, demonstrating a high sequence heterogeneity when comparing the four antibodies. To assess whether antigen binding is mainly mediated by the selected heavy chains, variants were produced with a different light chain, namely the one of EGFR-specific humanized Cetuximab (huC225). Binding analyses against CEACAM5 and CEACAM6 revealed that variants C5_A, C5_B (CEACAM5 selection) and C6_B (CEACAM6 selection) were incapable of binding their antigens when combined with huC225 light chain. Only variant C6_A (CEACAM6 selection) displayed similar binding kinetics to CEACAM6 compared to its progenitor with the common light chain (Supplementary data Fig. S6). Moreover, to verify occurrence of binders also in the least abundant sequence clusters, we exemplarily analyzed two additional CEACAM5 candidates from different clusters and measured their affinities. These candidates (harboring the IGKV3-15*01 common light chain) also displayed CEACAM5 specific binding with low $K_D$ (Supplementary data Fig. S7).

**Fig. 2** Enrichment analyses of CEACAM5 (C5) and CEACAM6 (C6) YSD immune libraries monitored by flow cytometry and sequence analyses within unselected and selected repertoires. (A) Flow cytometric analyses of CEACAM5 (C5; unselected and after three rounds of sorting) and CEACAM6 (C6; unselected and after two rounds of sorting) immune libraries with their respective antigens. Yeast cells were consecutively incubated with PE conjugated anti-kappa-antibody and His tagged CEACAM5 or CEACAM6 (500 nM), followed by secondary labeling with Alexa Fluor 647 conjugated anti-Penta-His antibody for simultaneous detection of Fab display and antigen binding. (B) CDR diversities were determined by clustering antibody sequences within CDRs to identities of $\geq 90\%$ ($n = 100$ sequences per unselected and selected library). Percentage values were calculated by dividing the resulting number of clusters by the total number of applied sequences. The number of identified clusters decreased with increasing enrichment.

**Generation of bsAbs with a common light chain**

The generation of IgG-like bsAbs with a common light chain requires a correct heterodimerization of the two distinct heavy chains. Therefore, we applied the SEED technology, which mediates specific heavy chain pairing via introduction of IgG- and IgA-CH3 strand exchanges. The resulting hybrid heavy chains (termed ‘AG’ and ‘GA’) form an asymmetrical CH3 interface which leads to the predominant formation of heterodimers (Davis et al., 2010). For the production of bispecific anti-CEACAM5/anti-CEACAM6 SEEDbodies, the heavy chain variable fragments of the yeast-
display selected common light chain Fabs were cloned into expres-
sion plasmids encoding either the SEED-AG (C5_A and C5_B) or
the SEED-GA (C6_A and C6_B) heavy chain Fc. The resulting
plasmids allowed the expression of four different bsAb variants
(C5_A × C6_A, C5_B × C6_A, C5_A × C6_B and C5_B × C6_B).
For the evaluation of monovalent antigen binding kinetics, all four
variants were also produced as ‘one armed’ (oa) SEEDbodies
(oaC5_A, oaC5_B, oaC6_A, oaC6_B) where only one heavy chain
comprises an antigen binding domain. Detailed kinetic analyses of
bispecific and corresponding oaSEEDbodies were measured using
BLI, exemplarily shown for antibodies C5_B × C6_A, oaC5_B and
oaC6_A (Fig. 4, Table 1). BLI analyses indicated that both
oaSEEDbodies (oaC5_B and oaC6_A) displayed exclusive binding
to their respective antigen, while the bispecific molecule (C5_B ×
C6_A) was capable of binding both antigens. Similar results were
obtained with all other generated monospecific and bispecific
SEEDbody constructs (Supplementary data Fig. S8–S10). Interestingly,
the combination of variant C5_A with either C6_A or C6_B led to a
significant affinity increase to CEACAM6 (Table 1). This observation
was probably due to a very slight binding of variant C5_A to
CEACAM6, which was not resolvable by BLI experiments but could
be demonstrated by ELISA (data not shown). Altogether, yeast-
display selected common light chain antibodies from OmniRat
immune libraries showed specific high affinity antigen binding, with
K_D values ranging between subnanomolar (C5_A; C6_B) and single-
digit nanomolar range (C5_B; C6_A). To ensure that the SEEDbodies
have similar biophysical properties as compared to conventional
IgGs, all generated variants were analyzed for aggregation and ther-
mal stability by size exclusion chromatography (SEC, Supplementary
data Fig. S11) and differential scanning fluorimetry (Supplementary
data Fig. S12), respectively. No enhanced aggregation behavior was
observed, while the thermal stabilities were only slightly decreased
(ΔT ≤ 3°C) compared to the IgG-molecules (Table 1). A general
slightly decreased thermal stability of SEEDbody molecules compared
to classical IgGs is in accordance with previous findings (Muda et al.,
2011). The utilization of a common light chain per se seems not to
affect the overall stability of the tested antibodies.

Characterization of SEEDbodies
To demonstrate that the generated bispecific common light chain
antibodies were able to bind both antigens simultaneously, BLI
experiments were performed with both antigens as analytes. For
that, all SEED-variants were subjected to two consecutive binding

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**Fig. 3** BLI sensorgrams of CEACAM5 or CEACAM6 binding to immobilized IgG-variants C5_A, C5_B and C6_A, C6_B. Association with 100 nM recombinant CEACAM5 or CEACAM6 was measured for 300 s followed by dissociation measurement for 600 s.
cycles in which immobilized antibodies were associated with recombinant CEACAM6 in the first cycle, followed by association with recombinant CEACAM5 or CEACAM6 at indicated concentrations was measured for 400 s while dissociation was measured for 900 s. Fitting of binding curves was calculated using a 1:1 binding model.

**Table I.** Thermal stabilities (Tm) and binding affinities (KD) of different antibody formats (IgG1, one armed SEEDbody, bispecific SEEDbody) of variants derived from yeast-display selected CEACAM5 and CEACAM6 immune libraries

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<td>C5_A × C6_A</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>C5_B × C6_A</td>
<td>66</td>
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</tr>
<tr>
<td>Tm [°C]</td>
<td></td>
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<tr>
<td>KD [nM]</td>
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<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>KD [nM]</td>
<td>0.3</td>
<td>1.3</td>
<td>0.2</td>
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<tr>
<td>KD [nM]</td>
<td>0.7</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>KD [nM]</td>
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<td>1.0</td>
<td>0.2</td>
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<tr>
<td>KD [nM]</td>
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<td>9.3</td>
<td>5.0</td>
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<tr>
<td>CEACAM5</td>
<td></td>
<td></td>
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<td>CEACAM6</td>
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Yeast selections were performed with recombinant soluble target protein. To assess whether the isolated common light chain Fabs were also able to bind to cellular expressed antigen, flow cytometric analyses were performed with all generated monospecific and bispecific SEEDbodies on cells transiently expressing CEACAM5 or CEACAM6. Stainings were conducted 2 days post transfection using a FITC conjugated anti-Fc antibody for detection (Fig. 6A,B). As expected, the bispecific molecules were able to bind to cells expressing either CEACAM5 or CEACAM6 (Fig. 6A,B; red lines), while the monospecific variants bound only to cells displaying their respective target (Fig. 6A,B; black and blue lines). To confirm that generated bispecific molecules were also able to bind to native antigen on tumor cells, flow cytometric analyses of the bispecific SEEDbodies were performed with the gastric cancer cell line MKN-45, expressing both, CEACAM5 and CEACAM6. All bispecific molecules displayed binding on MKN-45 while no binding could be observed to negative cell line Expi293 (Fig. 6C).
Generation and characterization of bsAbs with the light chain of an existing therapeutic antibody

Conceptually, any given light chain can be used as a ‘common light chain’ for the combination with a heavy chain diversity derived from immunizations of transgenic rodents. Therefore, the proposed methodology appears to be exceptionally suitable for the generation of bsAbs based on already existing therapeutic molecules. To this end, we combined the existing CEACAM5 and CEACAM6 immune libraries with the light chain of the anti-EGFR antibody huC225. For that EBY100 cells harboring CEACAM5 and CEACAM6 heavy chain diversities were mated with BJ5464 cells harboring the huC225 light chain resulting in library sizes of $6 \times 10^8$ (CEACAM5) and $5 \times 10^8$ (CEACAM6) individual clones, respectively. After two consecutive selection rounds by YSD specific binders could be enriched from both libraries, indicated by the yeast phenotype and sequence diversity analyses using the same clustering strategy as described above (Supplementary data Fig. S16, S17). From both campaigns, two variants with the highest prevalence were chosen from the most abundant sequence clusters and subcloned for expression as full-length IgG1 in combination with the huC225 light chain. After production in mammalian cell cultures, binding to recombinant CEACAM5 and CEACAM6 was again evaluated by BLI (Supplementary data Fig. S18). All four molecules showed specific binding to their antigen in the single-digit nanomolar range (Supplementary data Table S1). Specific cellular binding was proven via flow cytometry with transiently transfected Expi293 cells expressing either CEACAM5 or CEACAM6 and tumor cells expressing EGFR (Fig. 7C). SEC profiles (Supplementary data Fig. S23) and thermal stabilities (Supplementary data Fig. S24) indicated that biophysical properties of the bsAbs are in a reasonable range for potential therapeutic applications (Table I).

Discussion

The generation of IgG-like bsAbs is still a challenging task since it requires the correct assembly of two distinct heavy and two distinct light chains. While the specific heterodimerization of different heavy chains was intensively studied during the last decades, inhibition of random heavy/light chain association remains a demanding issue (Klein et al., 2012). Only a few examples for the enforcement of a specific heavy/light chain pairing are described in literature including the incorporation of mutations into interface residues of VL-CL and VH-CH1 or the domain crossover (CrossMab) technology (Merchant et al., 1998; Schaefer et al., 2011; Lewis et al., 2014). In CrossMab molecules specific heavy and light chain pairing is triggered e.g., by the exchange of CH1 and CL between the heavy and light chain on one half of the bsAb (Schaefer et al., 2011). Another obvious solution is the use of a common light chain on both binding arms of the...
antibody. Although this concept is mentioned in many reviews (Carter, 2001; Klein et al., 2012; Kontermann and Brinkmann, 2015), limited experimental data following this strategy have been published to date. Genentech presented a method to isolate such bsAbs from a large non-immunized phage display library with a restricted light chain repertoire. Despite the fact that libraries were not constructed to express a single light chain, binders against several targets were identified sharing the same light chains (Merchant et al., 1998). This work was extended by the generation of phage display libraries utilizing a fixed light chain and synthetic heavy chain diversities grafted into CD\(\mathrm{R}\)s of a human VH backbone. By application of this method it was possible to isolate common light chain antibodies against Fc\(\gamma\)RI\(\alpha\) and Fc\(\gamma\)RII\(b\) (Jackman et al., 2010). However, a high affinity common light chain antibody (KD: 0.6 nM) was only obtained after two rounds of affinity maturation. Similar results were observed by Sally Ward who combined a murine immune VH repertoire with a fixed light chain and identified one antigen-specific VH/VL pair (Ward, 1995) (KD: 120 nM) using phage display. Due to the utilization of a murine heavy chain diversity, selected binders from such an approach might not be applied as therapeutics due to immunogenicity issues.

In this study, we describe an approach for the efficient generation of a set of fully human bsAbs with common light chains. This approach relies on the isolation of immune evolved antibodies, which are obtained by immunization of transgenic rats that genetically encode human variable antibody domains (Bruggemann et al., 2015). Cloning of the human heavy chain encoding cDNA from B cells into the YSD format allows for the combination of the immune evolved heavy chain repertoire with any given light chain by yeast mating and the efficient selection of target specific variants via FACS. For proof of concept, we combined the light chain of IGKV3-15*01 germline origin with immune heavy chain repertoires derived from OmniRat (Ma et al., 2013; Osborn et al., 2013) immunizations with the cancer relevant antigens CEACAM5 and CEACAM6. After two to three rounds of sorting, we were able to identify a diverse set of high affinity binders against both targets, all utilizing the same light chain. Compared to other described YSD immune library approaches, the same or less selection rounds were required to obtain antigen-specific molecules (Weaver-Feldhaus et al., 2004; Bowley et al., 2007). This may indicate that the rat antibody repertoire contains a significant fraction of molecules, where the binding to the antigen is mainly driven by the heavy chain. Surprisingly, some isolated CEACAM5 and CEACAM6 binders displayed affinities in the picomolar range, indicating that neither affinity nor selectivity was compromised by utilizing the common light chain (Fig. 4). The antigen binding contribution of the light chain in an antibody paratope has been discussed in literature for years (Xu and Davis, 2000). Numerous previous studies indicate that antibodies lacking light chains can mediate affinity and specificity.

**Fig. 6** Cellular binding analyses of bispecific SEED and corresponding oaSEEDbodies. Binding to transiently transfected CEACAM5 or CEACAM6 expressing Exp293 cells and binding to cancer cell line MKN-45 cells was measured by flow cytometry. Of the note, 1 \(\times\) 10^5 cells were incubated with SEEDbodies followed by an incubation with an Alexa Fluor 488-conjugated anti-human IgG-antibody. (A, B) Black lines: CEACAM5 specific oaSEEDs; red lines: bspecific SEEDs. (C) Binding of bspecific SEEDs to MKN-45 cells. red lines: binding to MKN-45 cells; gray peaks: binding to Exp293 cells.
comparable to that of conventional mAbs (Muyldermans, 2013; Zielonka et al., 2015; Krah et al., 2016). The immune system of camelids and sharks contains such heavy chain only antibodies with their antigen binding domains designated as VHHs and vNARs, respectively (Krah et al., 2016). However, these single domain antibodies (dAbs) make up only a small fraction of the antibody repertoire of these animals which indicates an important contribution of light chains to a broad immune response. The transfer of the HCAb principle to human antibodies led to the development of the so-called dAbs, which closely resemble the architecture of single VH domains (VH dAb) (Holt et al., 2003). While the naturally evolved VHHs and vNARs show high solubility and physicochemical stability, human dAbs revealed a high aggregation tendency and poor solubility with the requirement of extensive protein engineering (Kim et al., 2014). These issues can be omitted by combining VH domains with common light chains. We found that only one out of four antibodies (C6_A) allowed for the exchange of the initially selected common light chain against a different one without loss of binding (Supplementary data Fig. S7). Hence, it is tempting to speculate that heavy chain variants C5_A, C5_B and C6_B were evolved by the rat immune system paired to a light chain that is similar to the utilized common light chain IGKV3-15*01. Nevertheless, the library also contained VH/VL pairs, where the heavy chain alone mediated high affinity antigen binding. This was the case for variant C6_A, which displayed identical affinity to CEACAM6 irrespective of the nature of the associated light chain. In this respect, the so-called DutaMab technology developed by Roche employs an alternative antibody format in which the diversity of only three CDRs is used to mediate antigen binding. Bispecific molecules can be generated by pairing an heavy chain specific for a particular antigen with a light chain specific for another cognate antigen (Beckmann, 2012). The heavy chain of antibody C6_A might be taken into consideration for a DutaMab approach.

Human common light chain antibodies could also be obtained by immunization of transgenic rodents that produce antibodies with a fixed single light chain which is currently followed by companies like OmniAb and Merus (Dhimolea and Reichert, 2012; Bruggemann et al., 2015). However, this could not be applied for

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**Fig. 7** Analyses of simultaneous binding and cellular binding of monospecific and bispecific SEEDbodies. BLI analyses to assess the simultaneous binding of bispecific huC225 light chain SEEDbodies (A) and cellular binding analyses of bispecific SEED and corresponding oaseEDbodies on CEACAM5 or CEACAM6 transiently transfected Expi293 cells and MDA-MB-231 cells (B) measured by flow cytometry. (A) Bispecific huC225 light chain SEEDbody C5_C × huC225 associated with CEACAM5 and EGFR. (B) Bispecific huC225 light chain SEEDbody C6_C × huC225 associated with CEACAM6 and EGFR. (C) 1 × 10^5 cells (CEACAM5 Expi293; CEACAM6 Expi293; MDA-MB-231) were incubated with SEEDbodies followed by an incubation with an Alexa Fluor 488-conjugated anti-human IgG-antibody. Black lines: CEACAM5 (upper row) and CEACAM6 (lower row) specific oaseEDs; blue lines: EGFR-specific huC225 oaseED; red lines: bispecific SEEDs.
the integration of existing antibodies into a bsAb format. It will be interesting to learn, whether our finding that only a fraction of the heavy chain antibodies tolerate light chain exchange, also prevails for antibodies obtained from rodents with a germline-encoded common light chain. Our data indicate that binder diversity does not depend on the choice of the common light chain, since the combination of the heavy chain immune repertoires with the light chain of huC225 (which shares only ~67% of sequence identity with IGKV3-15*01) also led to high affinity binders (Supplementary data Fig. S18). As expected, variant C6.A was enriched during the screening of the CEACAM6 heavy chain diversity combined with either the IGKV3-15*01 or the huC225 light chain. Obtained binders were subcloned into bsAb format utilizing the SEED-technology. All generated CEACAM5xCEACAM6 and CEACAM5xEGFR, CEACAM6xEGFR bsAbs displayed high affinity and specificity as well as specific cellular and simultaneous binding to both targets. A complementary approach was described recently for the generation of bsAbs aimed at combining κ or λ light chain repertoires from semisynthetic or naive libraries with a common heavy chain. Utilizing phase display pannings, binders against several antigens were obtained, which were then used for the construction of bispecific ‘κλ’-bodies (Fischer et al., 2015). Although this is a very elegant approach to generate bsAbs, only 50% of produced antibodies in cell culture is the correctly assembled κλ-body, while also κκ and λλ monospecific variants with identical arms are formed (Fischer et al., 2015).

In conclusion, we have established a procedure for the generation of bsAbs based on heavy chain binder selection of immune libraries in combination with any given common light chain. This facilitates the generation of bsAbs sharing a common light chain, where one binding arm is readily available as exemplified for the humanized version of the therapeutic antibody Cetuximab (huC225). Furthermore, the combination of in vivo immunization with an in vitro selection method eludes the issue of low fusion efficiency connected with classical hybridoma technology and allows for efficient multi-parameter high-throughput selection via FACS. It is important to note that the target-binding combinations programmed into the common light chain bsAbs described here, were chosen for a technological proof of concept study without any consideration of a potential clinical benefit. It should also be noted that any heavy chain heterodimerization technology can be utilized to generate this type of common light chain bsAbs. A very promising concept for the application of bsAbs relies on targeting a tumor specific antigen together with the recruitment of T effector cells. The first bsAb reaching market approval follows this strategy by binding to EpCAM and CD3 (Davis et al., 2010; Kontermann and Brinkmann, 2015). It will be interesting to learn in the future, whether common light chain SEEDbodies provide a new class of bsAbs with clinical benefits for immunotherapeutic and other applications.

**Supplementary data**

Supplementary data are available at Protein Engineering, Design & Selection online.

**Acknowledgements**

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**References**


