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The Shark Strikes Twice: Hypervariable Loop 2 of Shark IgNAR Antibody Variable Domains and Its Potential to Function as an Autonomous Paratope
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Short communication

The shark strikes twice: Hypervariable loop 2 of shark IgNAR antibody variable domains and its potential to function as an autonomous paratope

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Keywords

Antibody engineering, antibody technology, shark, IgNAR, single chain binding domain, heavy chain antibody, yeast surface display, antigen-binding site

Abstract

In this present study we engineered hypervariable loop 2 (HV2) of the IgNAR variable domain in a way that it solely facilitates antigen binding, potentially functioning as an autonomous paratope. For this, the surface exposed loop corresponding to HV2 was diversified and antigen-specific vNAR molecules were isolated by library screening using yeast surface display (YSD) as platform technology. An EpCAM (Epithelial cell adhesion molecule)-specific vNAR was used as starting material and nine residues in HV2 were randomized. Target-specific clones comprising a new HV2-mediated paratope were isolated against cluster of differentiation 3ε

(CD3ε) and human Fcγ while retaining high affinity for EpCAM. Essentially, we demonstrate that a new paratope comprising moderate affinities against a given target molecule can be engineered into the vNAR scaffold that acts independent of the original antigen-binding site, composed of complementarity determining region 3 (CDR3) and CDR1.

Introduction

Besides antibodies with the classical composition of heavy and light chains, sharks produce a heavy-chain only homodimer, referred to as IgNAR, in which antigen binding is mediated by a single variable domain, named vNAR (Greenberg et al. 1995). Due to their high affinity and specificity in conjunction with their small size and high physicochemical stability, vNAR domains emerged as promising molecules for biomedical and biotechnological applications and consequently a plethora of antigen-specific vNAR molecules were isolated for various applications, as recently reviewed (Zielonka et al. 2014a). The vNAR domain displays several peculiar characteristics. By virtue of the deletion in the framework-2-CDR2-region, vNAR is the smallest antibody-like antigen binding domain in the animal kingdom known to date, with a molecular mass of approximately 12 kDa (Barelle et al. 2009; Stanfield et al. 2004). As a consequence, vNAR domains have only two complementary determining regions CDR1 and CDR3, the latter region primarily mediates antigen-binding. After antigen contact high rates of mutations cluster to the CDRs, to the CDR2 truncation site, where the remaining loop forms a belt-like structure at the bottom of the molecule and to a loop which corresponds to HV4 in T-cell receptors (Fig. 1). Correspondingly, these mutation-prone regions have been named HV2 and HV4, respectively (Dooley et al. 2006; Zielonka et al. 2014a). vNAR domains can be categorized into four types, based on the presence and absence of non-canonical cysteine residues, which are not found in classical antibody variable domains (Diaz et al. 2002; Kovalenko et al. 2013; Stanfield et al. 2004; Stanfield et al. 2007; Streltsov et al. 2005; Zielonka et al. 2014a). Except for type I domains, HV2 is located distantly from the conventional antigen-binding site, composed of CDR3, CDR1 and HV4 (Fig. 1). However, for type I vNAR molecules, CDR3 is held tightly into the direction of HV2 (Stanfield et al. 2004). Consistent with this structural feature, mutations are favored in this loop for this particular type of domain, indicating that in this context HV2 might participate in antigen binding (Flajnik et al. 2011; Stanfield et al. 2004).

Within this work, we set out to engineer HV2 in a type IV vNAR in a way that it solely facilitates antigen binding, independent from the conventional antigen-binding site composed of CDR3 and CDR1 (and HV4). For this, we randomized nine residues of HV2 of a high-affinity

EpCAM-binding semi-synthetic vNAR molecule, known as 5005 (Zielonka et al. 2014b). This particular molecule was isolated from a semisynthetic library where the regions corresponding to CDR1 and CDR3 were randomized. Hence, HV2 is not involved in antigen binding. Herein we report the isolation of antigen-binding vNAR molecules with artificial HV2 loops against two different antigens, CD3ε and non-glycosylated human Fcγ, using yeast surface display. Interestingly, isolated binders retained high affinities against EpCAM, demonstrating that HV2 can potentially function as a paratope without compromising the structural and functional integrity of the vNAR scaffold. To the best of our knowledge, this approach for the generation of a new antigen-binding site has not been reported to date.

Materials and Methods

Media and reagents

YPD medium contained 20 g/L tryptone, 20 g/L dextrose and 10 g/L yeast extract. SD-CAA medium was composed of 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L ammonium sulfate, 5 g/L Bacto casamino acids, 20 g/L dextrose, 8.6 g/L NaH_2PO_4 x H_2O, and 5.4 g/L Na_2HPO_4. SG-CAA medium was prepared similarly except for the substitution of 20 g/L dextrose by galactose. Additionally 10 % (w/v) polyethylene glycol 8000 (PEG 8000) was incorporated. Phosphate-buffered saline (PBS) contained 8.1 g/L NaCl, 0.75 g/L KCl, 1.13 g/L Na_2HPO_4 and 0.27 g/L KH_2PO_4, pH 7.4.

Recombinant human His-tagged EpCAM and recombinant human CD3ε were purchased from AcroBiosystems. Glycosylated human Fcγ was produced in-house. Non glycosylated human Fcγ, produced in-house, carries an Asn297Ala mutation, preventing glycosylation.

Library construction

pCT plasmid, carrying genetic information for EpCAM-specific vNAR molecule 5005 (Zielonka et al., 2014a) was used as template for HV2-randomized library construction. HV2-diversification was executed in a consecutive 2-step splicing by overlap extension polymerase chain reaction (PCR) as depicted in Fig. 2A. For all reactions the conditions were: 94 °C for 2 min, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C, followed by 72 °C for 7 min. Primer sequences are listed in Tab. S1. For the first PCR step, two reactions were carried out in parallel, each containing isolated plasmid DNA as starting material. In one reaction, primer pair HV2_SOE_rand_up/pCT_Seq_lo was used. The forward primer randomized 9 residues in the surface exposed loop, corresponding to HV2. Residues considered for randomization are shown in Fig. 1. In the other reaction, primer pair pCT_Seq_up/HV2_SOE_lo was engaged.

The respective PCR products were purified using Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. For the subsequent PCR, 1 µl of each PCR product was used as template, respectively. After 6 cycles, primer pair pCT_Seq_up/pCT_Seq_lo was added. The resulting PCR product was purified *via* Wizard® SV Gel and PCR Clean-up System.

The pCT plasmid (Boder and Wittrup 1997), used for gap repair cloning and surface presentation of vNAR library candidates, was digested with *Nhe*I and *Bam*HI and purified *via* Wizard® SV Gel and PCR Clean-up System. For electroporation, 1-2 µg of the digested plasmid and 6-8 µg of insert were used. After 1 h incubation at 30 °C (1:1 YPD and 1 M sorbitol) library size was calculated by dilution plating. Yeast cells (EBY 100) were transferred into SD-CAA medium. Stocks were stored at -80 °C. For yeast surface display cells were grown overnight at 30 °C in SD-CAA medium, transferred into SG-CAA medium and incubated for 1-2 days at 20 °C.

Binding assays on the yeast surface and library screening for the isolation of target-specific vNAR molecules

Procedures and protocols for yeast surface display of vNAR domains have already been described by our group (Zielonka et al. 2014b) and thus are herein only briefly described. Flow cytometry was used to analyze presentation on the yeast surface and for single clone analysis. About 10^7 cells were labeled consecutively with anti-cMyc antibody (monoclonal, mouse, made in-house) and anti-mouse IgG FITC conjugate (goat, Sigma Aldrich, diluted 1:10 in PBS) for at least 10 min on ice. For single clone analysis, vNAR-presenting cells were either incubated with biotinylated, His-tagged CD3ε or Fcγ antigen for 15 min on ice and subsequently stained with Steptavidin-APC (diluted 1:10), Penta-His Alexa Fluor 488 conjugate (Qiagen, diluted 1:30) or anti-Hu IgG (Fcγ-specific) PE (eBioscience, diluted 1:30) for 10 min. Plasmid DNA from positive clones was isolated and sent out for sequencing with pCT-seq_lo or pCT-seq_up oligonucleotide (Tab. S1).

Affinities of isolated vNAR variants on yeast cells were determined as described (Chao et al. 2006; VanAntwerp and Wittrup 2000; Zielonka et al. 2014b). Flow cytometric analysis was performed to determine antigen binding. At least eight different antigen concentrations were used and cells were incubated for at least 1 h with the respective antigen. For the final calculation, RFU was plotted against antigen concentration.

Library screening for the isolation of antigen-specific vNAR molecules was performed on a MoFlo cell sorter (Beckman Coulter) and analyzed *via* Summit 4.3. For two-dimensional

screening, cells were resuspended in PBS containing the desired concentrations of both antigens and incubated on ice for at least 30 min. Subsequently, antigen-binding was detected using streptavidin-allophycocyanin conjugate (diluted 1:10), Penta-His Alexa Fluor 488 conjugate, anti-Hu IgG (Fcγ-specific) PE or Fcγ-specific Fab-fragment conjugated to Alexa Fluor 488 (Jackson ImmunoResearch, diluted 1:30). For the first rounds of sorting of the initial library, approximately 2 x 10^8 cells were analyzed and sorted. Consecutive rounds were at least performed with a 10-fold excess of cells that were collected in the previous round to ensure coverage of the enriched population.

Results and Discussion

The artificial HV2-randomized library for yeast surface display was constructed by polymerase chain reaction (PCR) using plasmid encoded EpCAM-specific clone 5005 as starting material. In the framework of this clone, 9 residues in HV2 were totally diversified by the incorporation of trinucleotide mixtures encoding all 19 amino acids except cysteine into the corresponding oligonucleotide. Residues considered for library design are given in Fig. 1 (blue residues). The library was established in a consecutive 2-step splicing by overlap extension PCR, as depicted in Fig. 2A and a yeast surface display library with an estimated diversity of approximately 1 x 10^9 unique clones (calculated by dilution plating) was constructed in a homologous recombination-based process referred to as plasmid gap repair (Benatuil et al. 2010). As already described (Zielonka et al. 2014b), library encoded HV2-randomized vNAR fragments were displayed on the yeast surface *via* Aga1p/Aga2p association (Boder and Wittrup 1997). vNARs were expressed as fusion protein with HA-tag and cMyc-epitope at the *N*-terminus and *C*-terminus, respectively, for the detection of surface displayed library candidates. Surface presentation as well as maintenance of structural integrity i.e. EpCAM-binding of HV2-randomized library candidates was assessed by indirect fluorescence labeling of the cMyc-epitope or *via* EpCAM binding assays on the yeast surface (Fig. 2B and C). Within one day post induction, there was more than 40 % cMyc-tag expression of the library detectable and about 36 % of EpCAM labeling, indicating that the vast majority of displayed vNAR fragments retained their structural integrity after randomization of HV2. These findings are consistent with the observation that HV2 is a mutation-prone region (Dooley et al. 2006; Flajnik et al. 2011; Stanfield et al. 2004), potentially tolerating a high degree of diversity at the sequence level as well as structural multiplicity.

To evaluate whether this loop can potentially function as a distinct antigen-binding site, the library was screened against two different targets, CD3ε and non-glycosylated human Fcγ. To

isolate binders, targeting a new antigen with HV2 while retaining affinity against EpCAM, first and last round of screening were performed two-dimensionally for target-binding (Fig. 3). To this end, vNAR binding of his-tagged EpCAM was detected using an Alexa Fluor 488 labeled anti-His-tag antibody. Binding to CD3ε was detected using biotinylated antigen as well as Streptavidin-APC. To avoid off-target binding against detection reagents, the labeling strategy was alternated from using biotinylated CD3ε to his-tagged antigen which was detected via Alexa Fluor 488 labeled anti-His-tag antibody. Consequently, we only sorted for CD3ε-binding. Initial FACS sorting rounds were performed with 1 µM EpCAM and 2 µM CD3ε. To enhance screening stringency, hence, to obtain binders against CD3ε displaying adequate affinities, the respective target protein concentration was reduced to 1 µM in rounds 3 and 4. As shown in Fig. 3, we were able to enrich cells displaying a double-positive antigen-specific signal for EpCAM and CD3ε within four rounds of FACS sorting. Interestingly, we also enriched for a population which lost its ability for EpCAM binding, indicating that this particular population lost its structural integrity (Fig. 3).

Likewise, sorting carried out against non-glycosylated human Fcγ protein also revealed a significant enrichment of FACS double-positive clones (Fig. S1). In these particular screening experiments, binding to human Fcγ was detected using either anti-human Fcγ-specific IgG conjugated to PE (rounds 1,3 and 4) or Fcγ-specific Fab-fragment conjugated to Alexa Fluor 488 (sorting round 2).

Single clones were analyzed in terms of their ability to target both antigens, EpCAM and CD3ε, and double-positive clones were sent out for sequencing, resulting in the identification of several double-positive clones (Fig. S2). Importantly, all identified clones were direct progenies of parental molecule 5005, each comprising a unique sequence in HV2, resulting from library design. Most of the clones still bound strongly to EpCAM at a concentration of 1 µM but binding to CD3ε was very weak at 1 µM, as exemplarily shown for single clone H5 (Fig. S3). Notwithstanding, for one clone, referred to as clone B1 we observed binding to CD3ε to a significantly higher extent compared to all other analyzed clones, whereas for parental molecule 5005 no binding was observed against CD3ε at the highest concentration tested (Fig. 4). Since it was evidenced that yeast surface display allows for the instantaneous characterization of isolated binders in terms of affinity and stability without the need for soluble expression (Doerner et al. 2014; Gai and Wittrup 2007; VanAntwerp and Wittrup 2000; Zielonka et al. 2014b) we decided to determine equilibrium binding constants *via* affinity titration on cells, as given in Tab. 1. Binder B1 displayed affinities for CD3ε of about 400 nM, without significantly compromising the affinity against EpCAM, compared to its parental molecule 5005.

Importantly, selectivity assays conducted on the yeast surface revealed that no nonspecific binding was observable against unrelated target proteins **(Fig. S4).**

Akin to these observations, one double positive EpCAM- and human Fcγ-binding clone, referred to as clone F1 was identified after single clone analysis (Fig. S2). Characterization of this clone resulted in a moderate affinity against human Fcγ of approximately 2.6 µM. Furthermore, no significant off-target binding was detected against unrelated target proteins **(Fig. S4).** Affinity against EpCAM was comparable to that of its parental clone 5005, which does not display detectable binding to human Fcγ, as listed in Tab. 1. In this respect, no efforts were made to optimize affinities against Fcγ *via* alanine-scanning and sub-library screening. Interestingly, this clone only bound to non-glycosylated human Fcγ. For the glycosylated equivalent we were unable to detect any binding at the highest concentrations tested (Fig. 5). Hence, one can draw conclusions with regard to the epitope that is addressed by clone F1. It is tempting to speculate, that position 297 of Fcγ is involved in the vNAR-target-interaction, since for wild-type Fcγ, this residue is Asn which is typically glycosylated. However, unglycosylated Fcγ contains an Asn to Ala mutation at this position and it can be assumed that this mutation, that prevents glycosylation, is part of the epitope targeted by HV2-diversified clone F1. Another hypothesis is that glycosylation of the Fc-fragment covers the epitope targeted by α-EpCAM-Fcγ-vNAR F1 in a way that this region as structurally not accessible.

The immunoglobulin family displays a paramount tolerability in loop length as well as sequence variation, which is most evident for CDR regions of variable domains. However, this feature is a general hallmark of the immunoglobulin domains (Halaby et al. 1999; Wozniak-Knopp et al. 2010) and loop regions other than the natural CDRs can be considered for engineering novel binding characteristics. In this respect, surface-exposed loops at the N-terminal tip of CH2 of human IgG have been engineered for antigen binding (Xiao et al. 2009). In another very elegant approach, established by Rüker and co-workers, a new antigen-binding site was introduced in loop regions of the CH3 domain of human IgG (Wozniak-Knopp et al. 2010). Most importantly, those engineered Fc fragments, named Fcab, retained the ability to elicit effector functions, clearly indicating the structural integrity of the molecule.

Essentially, our study demonstrates that the vNAR scaffold can be engineered in a way that HV2 potentially functions as an independent paratope, exclusively facilitating antigen binding against a target-protein. Importantly the establishment of a new antigen binding site does not impair the conventional antigen-binding site, composed of CDR3 and CDR1 in its affinity. However, it needs to be mentioned that affinities mediated by HV2 are only moderate and almost certainly require optimization for further studies such as effector cell recruitment assays.

In this respect, affinity maturation might be performed by second generation randomization of the HV2-adjacent surface exposed loop, referred to as EF-loop, as already described by Wozniak-Knopp *et al.* for human constant CH3 domains (Wozniak-Knopp et al. 2010). This group utilized the EF-loop in CH3 as part of a novel antigen-binding site.

In recent years, bispecific antibodies and antibody domains have opened new avenues for the treatment of various diseases (Kontermann 2012; Weidle et al. 2014). Ultimately, it remains to be elucidated, whether shark-derived antibody domains may contribute to this continuously evolving field of therapeutic drug design in the future.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

Acknowledgments

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Figures and Tables

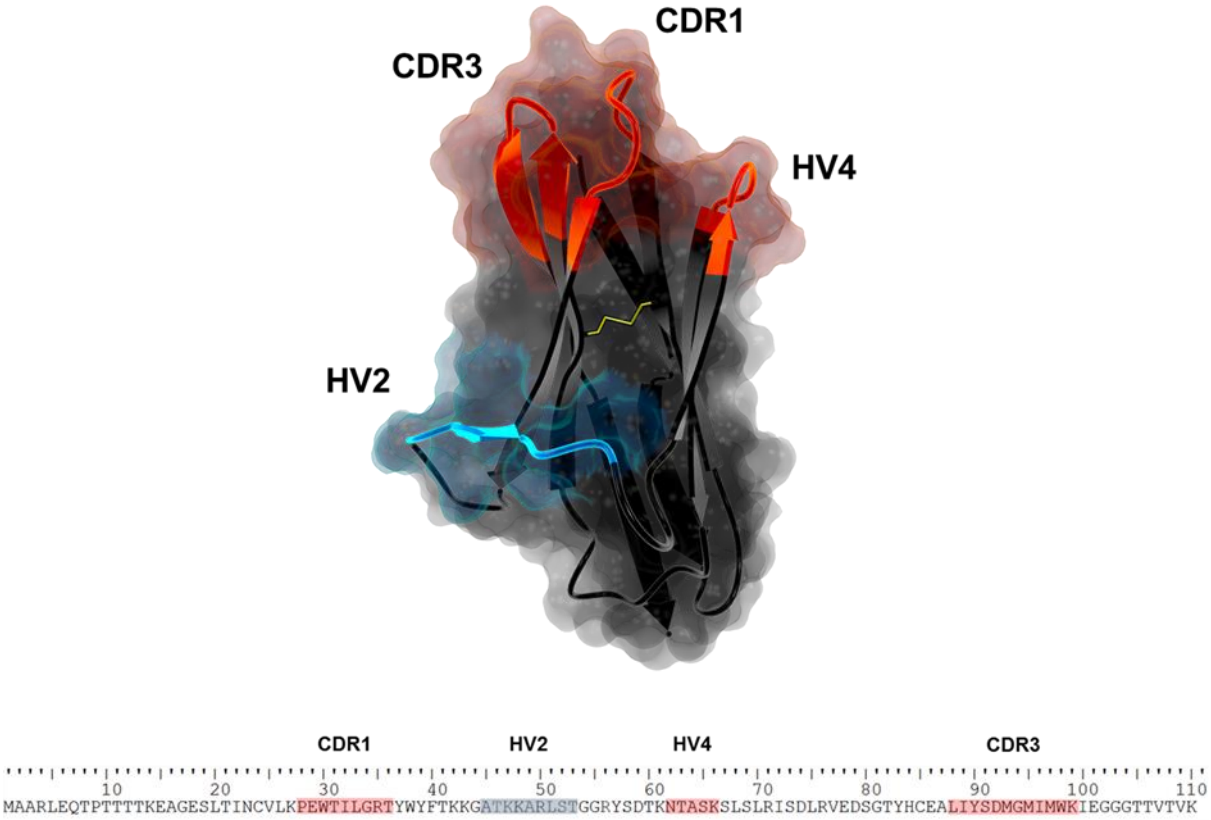


Fig. 1 Depiction of the rationale for the generation of a new antigen-binding site into the vNAR scaffold. The conventional paratope composed of CDR3, CDR1 (and HV4) is shown in red. The potentially new antigen-binding site, consisting of HV2 is shown in blue. Yellow: Disulfide bond. Model based on pdb entry 4HGK (Kovalenko et al. 2013) generated using YASARA structure.(Krieger et al. 2009) Sequence shown for parental molecule 5005 used for library design. Sequence for CDR1 and CDR3 as well as HV4 of the conventional antigen-binding site shaded in red. Residues in sequence exposed loop corresponding to HV2 and considered for randomization shown in blue.

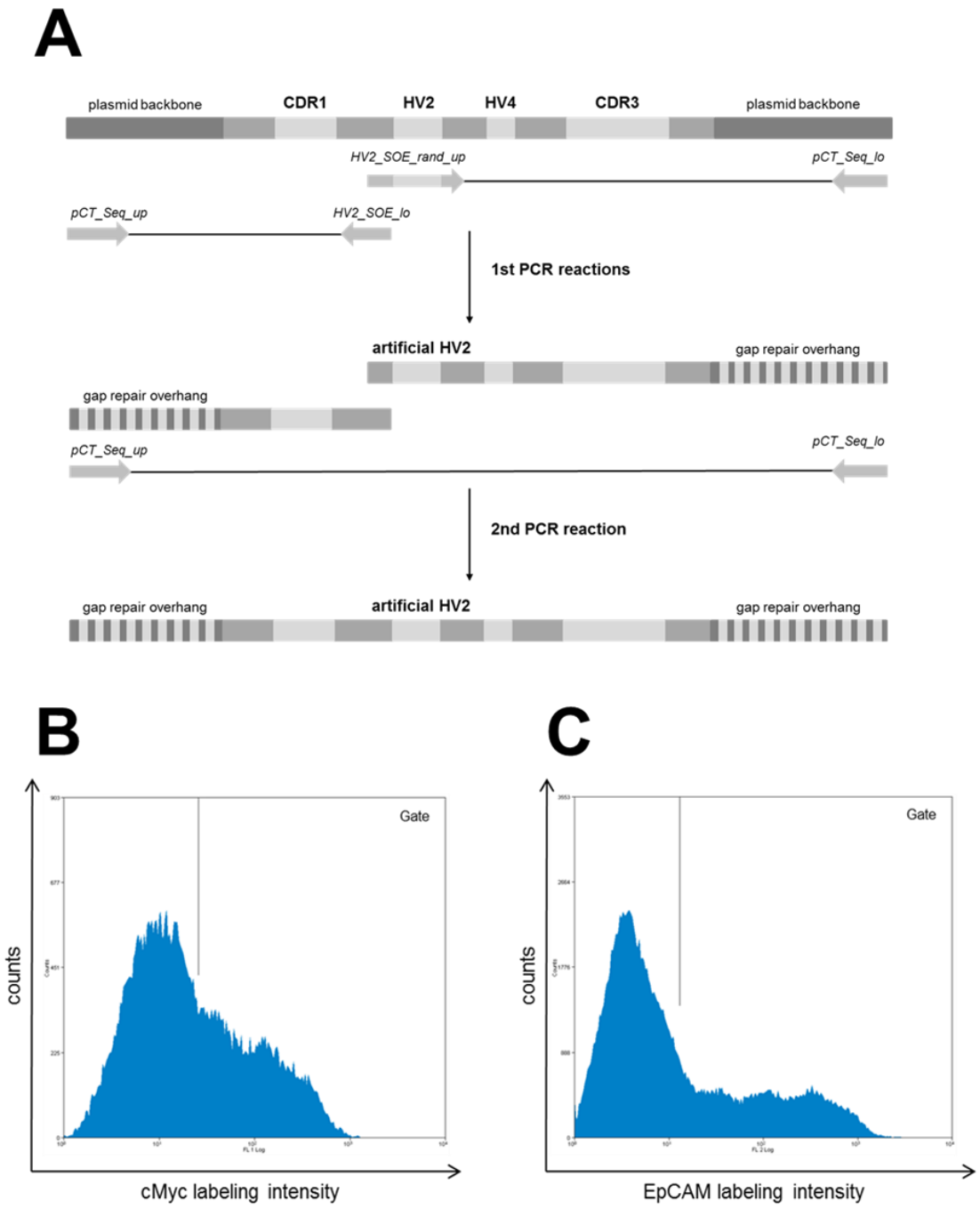


Fig. 2 (A) Schematic representation of PCR-based randomization of HV2. pCT plasmid encoding EpCAM-specific vNAR 5005 was used as template. In first PCRs HV2 was randomized and the corresponding up and low fragments of the vNAR domain were constructed. In a subsequent reaction the total vNAR molecule was constructed *via* splicing by overlap extension. (B and C) Analysis of HV2-randomized library based on EpCAM-specific vNAR 5005. Histogram of cMyc surface expression (B) and EpCAM-binding (C) of the constructed vNAR library assessed by indirect immunofluorescence labeling and flow cytometry, one day post induction. Cells in gate: 40.1 % for cMyc staining and 35.9 % for EpCAM-labeling (1 µM EpCAM, detected with EpCAM-specific antibody clone HEA-125 conjugated to PE, Miltenyi Biotec).

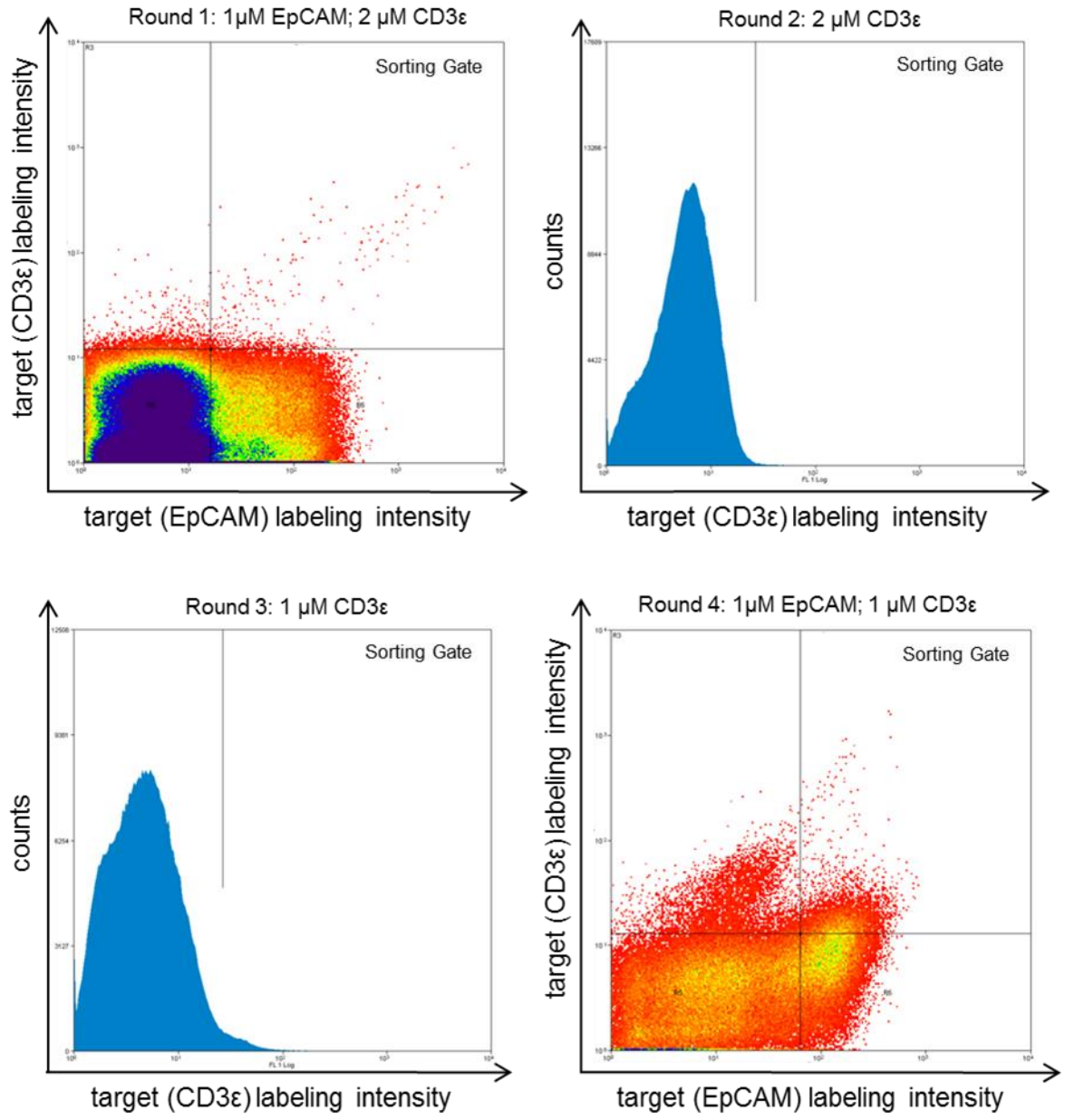
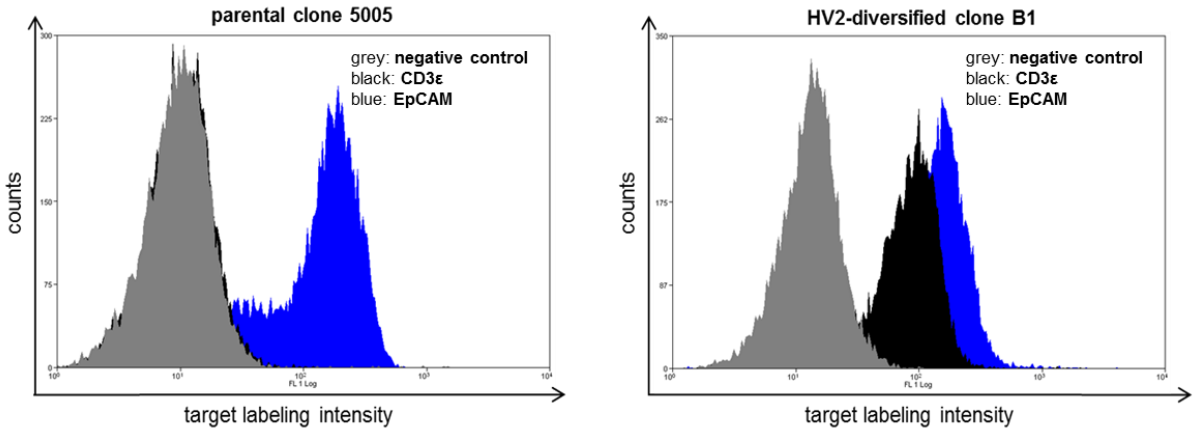


Fig. 3 Screening of a HV2-randomized EpCAM-specific library based on vNAR 5005 against CD3ε. Sorting gates and target concentrations are shown. In round one and four, cells were simultaneously labeled for EpCAM-binding using Penta-His Alexa Fluor 488 conjugate and CD3ε-binding *via* biotinylated antigen and Steptavidin-APC. In round two and three, cells were only labeled for CD3ε-binding using his-tagged antigen and Penta-His Alexa Fluor 488 conjugate. After round 3 a resort was performed. Cells in the sorting gate were isolated, grown and induced for the next round of selection. Target-positive cells in sorting gate: (R1) 0.1 %; (R2) 0.17 %; (R3) 1.92 % and (R4) 5.8 %.



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360 **Fig. 4** Single clone analysis of mono-specific, parental clone 5005 and HV2-diversified clone B1 for EpCAM and
361 CD3ε-binding. Single clones were incubated with 1 µM of the respective antigen. Alexa Fluor 488 labeled anti-
362 His-tag antibody was used for the detection of target binding. Blue: Cells stained with EpCAM. Black: Cells
363 incubated with CD3ε. Grey: Negative control, cells only labeled with secondary detection antibody.

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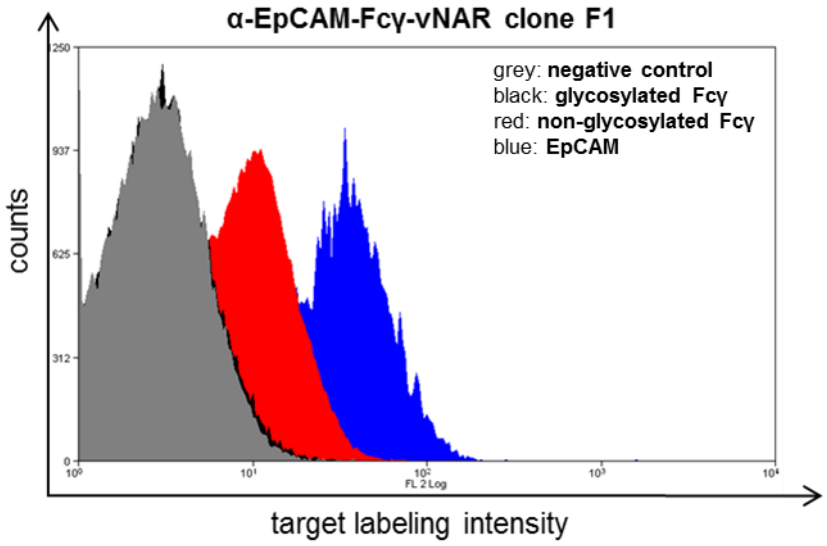

Fig. 5 Single clone analysis of EpCAM- and non-glycosylated Fcγ-binding HV2-diversified clone F1. Single clone was incubated with 1 µM of the respective antigen. Anti-human IgG conjugated to PE (Fcγ-specific) was used for the detection of target binding. Grey: Negative control, cells only labeled with secondary detection antibody. Black: Cells incubated with glycosylated Fcγ. Red: Cells labeled with non-glycosylated Fcγ. Blue: Cells labeled with EpCAM and detected *via* EpCAM-specific antibody clone HEA-125 conjugated to PE (Miltenyi Biotec).

374 **Tab. 1** Equilibrium dissociation constants (K_D) determined by yeast surface display for HV-diversified clones

375 compared to parental clone 5005.

vNAR clone	**Type of molecule**	**K_D [nM] EpCAM**	**K_D [nM] CD3ε/Fcγ**
5005	Parental molecule	40 ± 13	- (CD3ε/ Fcγ)
B1	After library screen **CD3ε**	46 ± 8	422 ± 60 (CD3ε)
F1	After library screen human **Fcγ**	53 ± 10	~ 2600 (Fcγ)

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