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Fab is the most efficient format to express functional antibodies by yeast surface display

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ABSTRACT

Multiple formats are available for engineering of monoclonal antibodies (mAbs) by yeast surface display, but they do not all lead to efficient expression of functional molecules. We therefore expressed four anti-tumor necrosis factor and two anti-IpaD mAbs as single-chain variable fragment (scFv), antigen-binding fragment (Fab) or single-chain Fabs and compared their expression levels and antigen-binding efficiency. Although the scFv and scFab formats are widely used in the literature, 2 of 6 antibodies were either not or weakly expressed. In contrast, all 6 antibodies expressed as Fab revealed strong binding and high affinity, comparable to that of the soluble form. We also demonstrated that the variations in expression did not affect Fab functionality and were due to variations in light chain display and not to misfolded dimers. Our results suggest that Fab is the most versatile format for the engineering of mAbs.

Introduction

Over the past 20 years, yeast surface display (YSD) has emerged as a very potent tool for protein engineering. It is a very proficient platform for *de novo* identification of new binders in naïve[1] or immune libraries.[2,3] YSD is also used for the engineering of many antibodies, principally to identify mutants with improved affinity[4,5] selectivity, stability and expression.[4-7] Affinity-matured antibodies have been developed against multiple antigens such as tumor necrosis factor (TNF),[8] fluorescein,[9] botulinum neurotoxin type A,[10] carcinoembryonic antigen,[11] epidermal growth factor receptor[12] and streptavidin.[13] Engineering of other properties of antibodies such as pH sensitivity or antigen specificity has also been described.[14,15]

Engineering of pre-existing antibodies using YSD relies on the anchoring of antibody fragments on the surface of yeast cells via fusion with a cell wall protein. Various formats are described in the literature for the functional display of antibody fragments on yeast cells, such as single-chain variable fragment (scFv), antigen-binding fragment (Fab) or single-chain Fab (scFab), although they are known to lead to variable levels of antibody expression.[2,4,10,16] Yeast display of IgG has also been reported with secretion and capture strategies,[17,18] but, to our knowledge, not as a conventional fusion with a yeast surface anchor protein. In most cases, antibody fragments are presented using the Aga2p-based display system, which permits either N-terminal or C-terminal fusion,[4] but the lack of comparative studies does not facilitate the choice of the appropriate formats to be used.

ScFv molecules are the smallest functional entity (30 kDa) that includes the complete antigen-binding site of an antibody. This entity comprises the two variables domains VH and VL connected by a flexible linker that prevents their dissociation.

Fab are composed of the light chain (VL + CL) and a heavy chain (VH + CH1) connected by an interchain disulfide bond. Several studies have reported the expression of Fab molecules on the surface of yeast cells using either a bigenic plasmid[13] or two distinct plasmids[10,14,19] to allow separate expression of the two chains. Expression levels of Fab molecules are generally considered as lower than those of scFv molecules in *E. coli*,[20,21] at the surface of phages[22,23] and also on the surface of yeast cells.[10]

The scFab derived from Fab is obtained through introduction of a polypeptide linker connecting the light chain and the heavy chain. Thus, it combines some advantages of the scFv (single chain and one expression plasmid, level of expression) with the stability and lower tendency to aggregate of the Fab.[2,23] The linker prevents the display of unpaired heavy chain antibodies (devoid of light chain), which can sometimes interfere with the selection steps.[2] ScFab molecules carry a flexible linker of variable length (32 to 80 amino acids) and an optional inter-chain disulfide bond.[2,24] Deletion of carboxy terminal cysteine residues (ΔC) of the CL and CH1 domains results in a scFabΔC format, which has increased expression levels in *E. coli*.[23]

The scFv is the most popular format for antibody engineering,[4] probably because of its very high level of expression, its small size and the presence of a single peptide chain, which is an advantage for cloning or sequencing procedures. However,

Fab and scFab formats have undeniable advantages, especially when it comes to stability or for reformatting to IgG molecules after engineering.[22] To date, there is no clear literature consensus regarding the choice of an antibody fragment format suitable for engineering using yeast cell display. Also, no general study has yet explored the influence of antibody fragment formats on a diversity of antibodies in this particular context.

Here, we report the comparison of 4 antibody fragment formats – scFv, scFab with and without interchain disulfide bond and Fab – for the functional display of 6 antibodies. This study includes a first set of well-known antibodies – adalimumab, infliximab, golimumab and certolizumab – already used in clinical settings. This set of antibodies encompasses humanized, chimeric and fully human antibodies, which share TNF as a common antigen.[25] The second set is composed of two chimeric antibodies targeting the protein IpaD from the needle tip of type III secretion system of *Shigella* spp obtained from mouse immunization. The objective of this study was to identify the most suitable format for the functional expression of antibody fragments on the surface of yeast cells.

Results

Conception of yeast surface display vectors allowing expression of scFv, scFab and Fab antibody fragments

To determine the influence of formats on the expression and functionality of antibody fragments, a new subset of yeast display plasmids was designed. Four plasmids were constructed for respective expression of scFv, scFab formats and Fab at the surface of *S. cerevisiae* cells. Those plasmids share a common backbone from previously described pCT-L7.5.1,[26] including inducible promoter *Gal 1–10* and Aga2p signal peptide [Fig. 1]. Antibody fragments were displayed with a free N-terminus and tethered to the yeast cell wall by C-terminal fusion with Aga2p,[27] in order to avoid possible steric hindrance of the fusion.

The plasmid pNT scFv encodes a typical scFv composed of VH/VL domains connected by a flexible linker $(G4S)_3$. We also compared two previously described scFab formats, scFabΔC[2] and sc60Fab,[24] by constructing the plasmids pNT scFabΔC and pNT sc60Fab. ScFabs are composed of a VL-C_K light chain connected to the VH-CH1 heavy chain by a flexible linker. The difference between the two formats resides in the nature and length of the linker (34 amino acids for scFabΔC versus 60

amino acids for sc60Fab) and the presence of an inter-chain disulfide bond in sc60Fab connecting the two C-terminal cysteine residues of C_K and CH1. Display of Fab molecules on yeast without any linker was achieved using the bigenic plasmid pNT Fab where *Gal 1–10* promoter and terminator are duplicated to obtain separate expression of the two chains.

Each plasmid includes a hemagglutinin (HA) tag to report expression of the complete antibody fragment. For single chain formats (scFv and scFabs), the HA tag is located between the antibody fragment and the Aga2p subunit. Full Fab expression can be controlled for pNT Fab through the expression of the light chain with the HA tag or by direct labeling with anti-C_K antibody. Expression of heavy chain can be revealed by the HIS tag located between the heavy chain and Aga2p.

The 6 selected antibodies are IgG1 molecules with C_K light chains. The sequences of TNF-specific antibodies were obtained from the IMGT database.[28] The mAbs IpaD_301 and IpaD_318 were obtained by mouse immunization [data not shown] and engineered to generate chimeric antibodies. These two antibodies derive from the same parental genes *IGHV1-14*01/ IGKV1-117*01* from *Mus musculus* and differ by only 7 and 8 mutations in the heavy chain and the light chain, respectively. Together, 24 plasmids corresponding to the sequences of 6 selected antibodies expressed in the 4 antibody formats were successfully cloned and transformed into yeast. An exhaustive analysis of the 24 constructs was performed in parallel to investigate the influence of the antibody fragment format on antigen binding.

Fab format grants functional expression of the largest number of antibodies

To assess the expression and functionality of the constructs, yeast cells were incubated with the appropriate biotinylated antigen at a concentration far above the expected K_D to reach quantitative binding of the antibody fragments to their antigen. When evaluated by fluorescence-activated cell sorting (FACS), cells expressing scFvs of adalimumab, certolizumab, golimumab and IpaD_318 exhibited a high level of fluorescence, indicating quantitative binding of the antigen [Fig. 2]. Weaker fluorescence levels were observed for IpaD_301 scFv, whereas no signal was observed for infliximab scFv, indicating antigen binding was weak or absent. Functionality of constructs displaying low or moderate antigen binding was not improved by increased
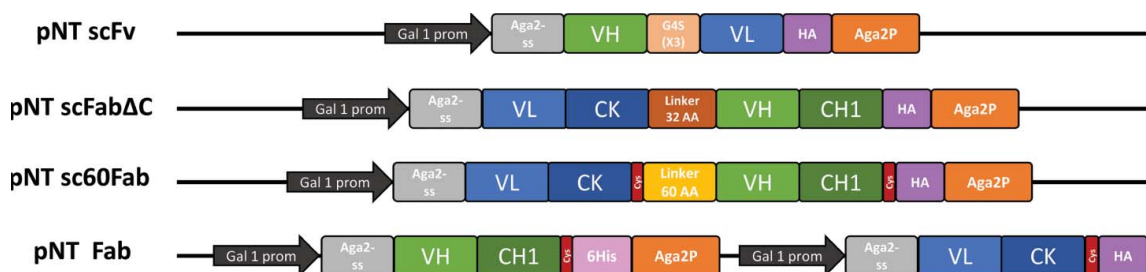
Figure 1. Schematic representation of yeast surface display vectors used in this study. Antibody fragment constructs are tethered to the cell wall of *S. cerevisiae* cells by C-terminal fusion with the Aga2p subunit of the a-agglutinin yeast cell surface anchor protein. Expression is controlled by the tightly regulated galactose-inducible *Gal1* promoter. Aga2-ss: native signal sequence of Aga2p for secretion of the constructs. Single-chain antibody fragments (scFv, scFabΔC and sc60Fab) include flexible linkers connecting the light chain and the heavy chain. pNT Fab is a bigenic plasmid including two identical GAL1 promoters for respective expression of light and heavy chains. Cysteine residues responsible for the formation of the inter-chain disulfide bond connecting C_K and CH1 are indicated in red. Expression of antibody fragments can be monitored using the HA tag for all constructs. A 6His-Tag sequence can also be used for the monitoring of heavy chain expression when using pNT Fab.
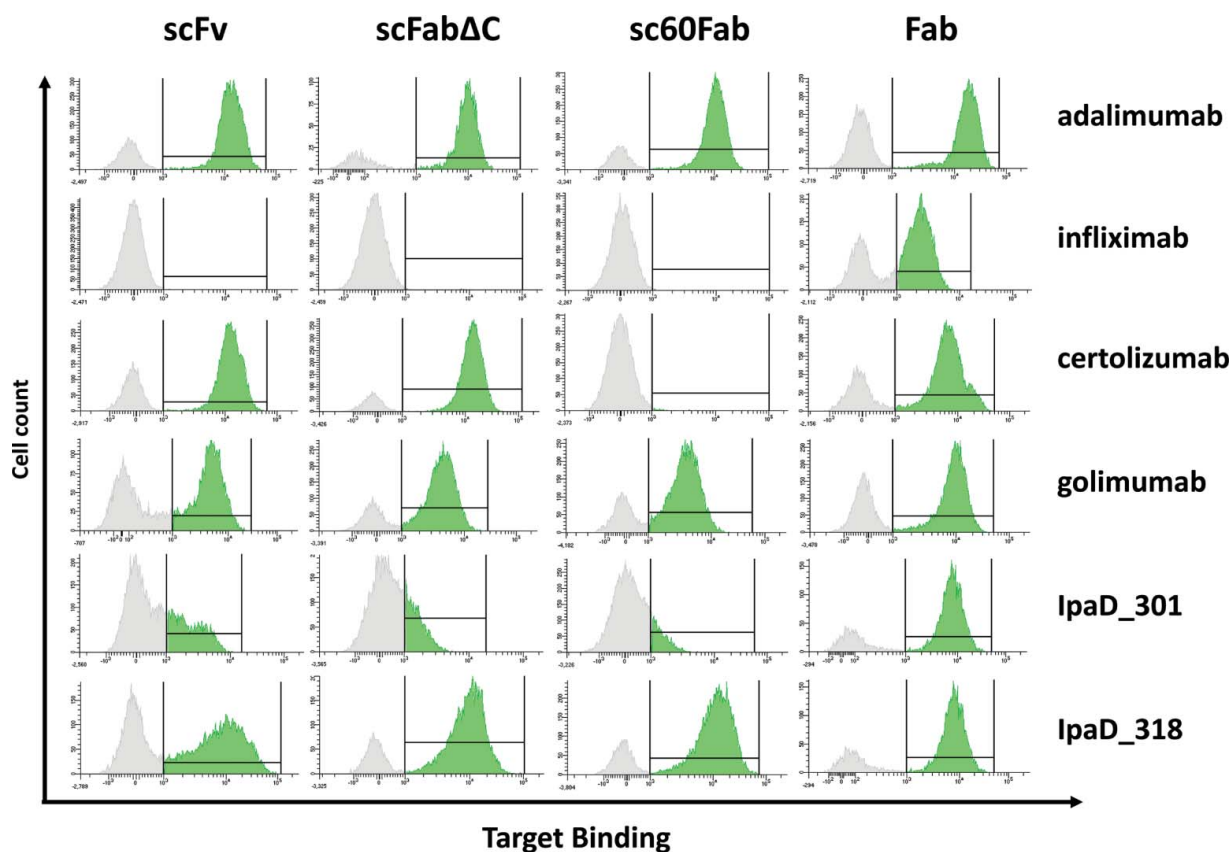
Figure 2. Antigen binding of the 6 selected antibodies expressed as different antibody fragment formats on the surface of yeast cells. Cells are labeled with biotinylated antigen then with Streptavidin–PE (Target Binding) before FACS analysis. Selected antigen concentrations are far above the expected K_D to reach a quantitative binding with the antibody fragments. Respective antigen concentration were 20 nM for anti-TNF antibodies and 50 nM for anti-IpaD antibodies. For each construct, a FACS histogram shows the fluorescence intensity corresponding to antigen binding (x-axis) vs the number of detected events (y-axis). Interval gates (bars) are shown to differentiate cells with detectable antigen binding (mean fluorescence intensity (MFI) score above 10^3, in green) from cells with no detectable antigen binding (MFI score under 10^3, in grey).The 10^3 cutoff has been obtained by measuring MFI of cells with a non-relevant antigen, cf. Supplementary figure 1.

induction times [Supp Data Fig. 3]. Antigen binding was specific, as demonstrated by the lack of cross-reactivity with the non-relevant antigen: anti-TNF antibodies showed no detectable IpaD binding and anti-IpaD antibodies showed no detectable TNF binding [Supp Data Fig. 1].

Yeast cells expressing scFab corresponding to adalimumab, golimumab and IpaD_318 displayed quantitative binding to their antigen, both as scFabΔC and sc60Fab formats. Infliximab and IpaD_301 in scFab formats were not or weakly functional, respectively, as attested by the absence of fluorescence for the corresponding cells. Interestingly, certolizumab scFabΔC showed significant binding to TNF, while certolizumab sc60Fab with the 60-amino-acid linker and inter-chain disulfide bond failed to bind TNF. All single-chain constructs were expressed at the surface of cells, as attested by labeling with anti-HA antibodies, with the notable exception of certolizumab sc60Fab [Supp Data Fig. 2]. Except for this specific construct, the absence of binding of the antibody fragments to their antigen could not be explained by lack of expression, but rather by a functional deficiency.

In contrast, all of the 6 tested antibodies bound to their antigen when expressed as Fab at the surface of yeast cells. For each antibody, cells expressing functional Fabs form homogeneous populations with a clear shift in fluorescence levels compared to non-expressing cells. The proportion of cells expressing functional Fabs is relatively high (from 46% to 74%).

In our set-up, Fab is the only viable format for functional expression of infliximab and IpaD_301 antibody fragments. Besides, for 5 of 6 antibodies tested, the fluorescence of cells expressing functional Fab was higher than the fluorescence of the equivalent cells expressing either scFvs or scFabs. This suggested that Fab molecules were not only active, but also well expressed. For these reasons, we focused on display of Fab molecules in the rest of the study.

Variations of expression in Fab format among antibodies do not affect functionality

We observed that maximal fluorescence levels of Fab-expressing cells vary among antibodies targeting a common antigen. More specifically, the mean fluorescence intensity of cells displaying functional infliximab Fabs was much lower than for adalimumab or golimumab, a phenomenon also observed for IpaD_301 when compared to IpaD_318.

As variations of signal corresponding to antigen binding could be attributed either to differences of expression levels or to functional differences, dissociation constants (K_D) were determined for each displayed Fab [Fig. 3]. The measured K_D values for IpaD_301 and IpaD_318 are 3.2 nM and 1.7 nM, respectively. This result is in accordance with the K_D evaluated by OctetRed with measured affinities of 1.1 nM and 0.9 nM
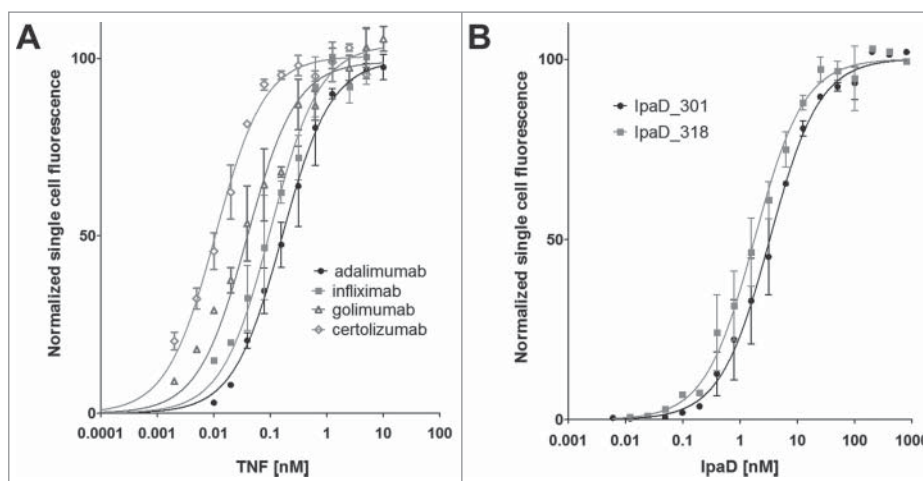
Figure 3. Affinity of Fab antibody fragments expressed on the surface of yeast cells. The mean fluorescence intensity (MFI), determined by FACS analysis, is plotted against varying concentrations of antigen for every Fab construct, and fit to a monovalent binding model. Measurements were done in triplicate with independent cultures and inductions. (A) Equilibrium binding constants K_D of the four anti-TNF Fabs for TNF at 20°C. Curve fitting results in a K_D of 114 ± 6 nM for adalimumab Fab, 102 ± 14 pM for infliximab Fab, 39 ± 4 pM for golimumab Fab and 11 ± 1 pM for certolizumab Fab. (B) Equilibrium binding constants K_D of the two anti-IpaD Fabs for IpaD at 20°C. K_D IpaD_301 Fab = 3.2 ± 0.3 nM, K_D IpaD_318 Fab = 1.7 ± 0.2 nM.

(Supplementary Fig. 4). Yeast expressing anti-TNF Fabs show subnanomolar K_D, 161 pM for adalimumab, 102 pM for infliximab, 39 pM for golimumab and 11 pM for certolizumab. These K_D values are consistent with the reported K_D determined for the corresponding IgG molecules using SPR,[25] indicating that Fabs expressed on the surface of yeast cells have a very strong affinity for their antigen.

Interestingly, higher affinity was not correlated with higher level of binding fluorescence. While having a very good affinity for TNF, infliximab Fab displayed much weaker TNF binding signal compared to adalimumab Fab, suggesting differences in their expression levels. Thus, additional labeling experiments were performed to evaluate different expression reporters. In addition to antigen binding labeling, light chain expression was
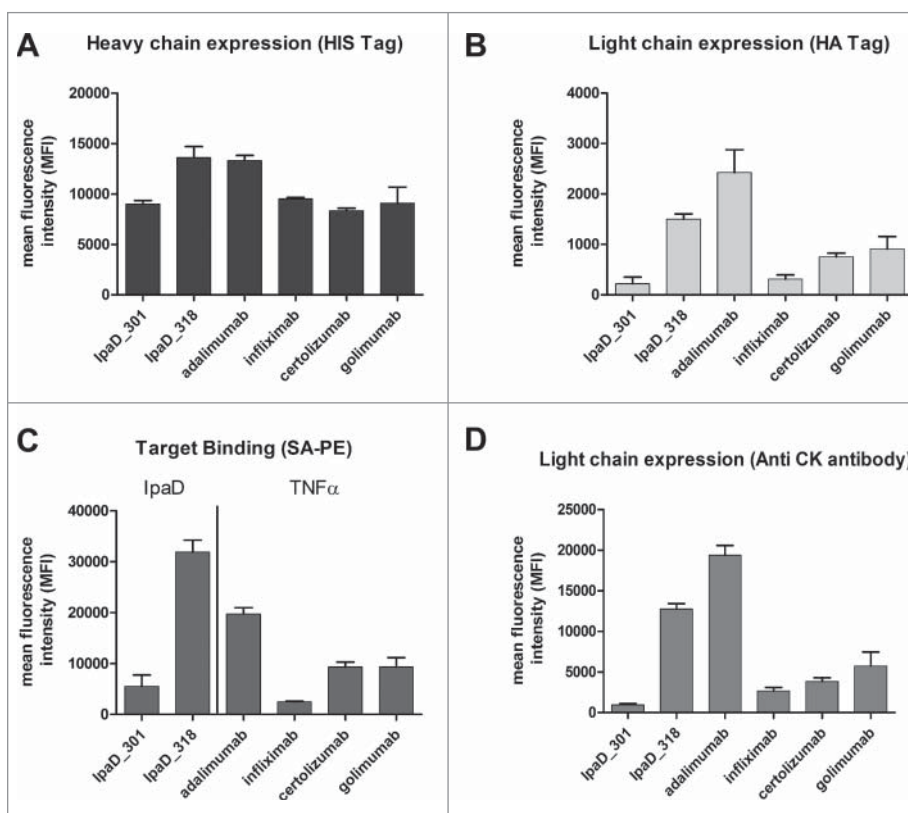
Figure 4. Antigen binding and expression levels of heavy and light chains of the 6 selected antibodies expressed as Fab on the surface of yeast cells. Cells were labeled with the appropriate expression reporter and the mean fluorescence intensity of positive cells was determined by FACS analysis. For antigen binding, cells were double-labeled with biotinylated antigen and Streptavidin–PE (target binding) before FACS analysis. (A) Heavy chain expression evaluated with anti-HIS antibody reporter. (B) Light chain expression evaluated with anti-HA antibody reporter (C) Biotinylated antigen binding evaluated with Streptavidin-PE (D) Light chain expression evaluated with anti-CK antibody reporter. Measurements were made in triplicate using cells from independent cultures.

evaluated with anti-HA or anti-C_K antibodies and heavy chain expression was assessed with anti-HIS antibodies [Fig. 4].

Heavy chain expression showed slight variations between the 6 selected antibodies [Fig. 4a]. Labeling with anti-HIS antibodies showed homogeneous expression of heavy chains, with IpaD_318 and adalimumab being somewhat above average. This observation contrasted with light chain expression, which showed strong differences across the Fab antibodies [Fig. 4b]. Light chain expression was significantly higher for IpaD_318 and adalimumab Fabs than for IpaD_301 or infliximab, while certolizumab and golimumab had intermediate light chain expression levels. Values observed for both reporters anti-HA or anti-C_K were in good agreement, and the relative intensities of constructs were very similar. Anti-C_K antibody yielded a very strong fluorescence signal, thus favoring easy discrimination of C_K-expressing cells.

Antigen binding signals for IpaD_301 and IpaD_318 correlated well with their relative light chain expression levels [Fig. 4c and Fig. 5]. Very similarly, the TNF binding signal was proportional to the respective expression levels of adalimumab, infliximab, certolizumab and golimumab light chain of the Fab

[Fig. 5]. Direct correlation between light chain expression and antigen binding was confirmed by simultaneous labeling with anti-C_K antibody conjugated to allophycocyanin (APC) and streptavidin R-phycoerythrin (streptavidin-PE) [Fig. 4d].

While having high sequence homology and similar affinities, IpaD_301 and IpaD_318 display significant differences in expression and antigen binding in all formats tested. For this reason, we investigated a possible correlation between the biophysical attributes of these two antibodies and their behavior on the surface of yeast cells. Thus, recombinant expression of complete IgG1 IpaD_301 and IpaD_318 was performed by transient transfection of HEK293 cells[29] followed by protein A purification. Production yield was slightly higher for IpaD_318 than for IpaD_301 (20 and 14 mg per liter, respectively). Size-exclusion chromatography revealed that both antibodies are predominantly produced as full-length IgG with low amounts of aggregates for IpaD_318 and even lower amounts for IpaD_301 [Fig. 6]. In contrast, differential scanning fluorimetry (DSF) analysis reveals that IpaD_301 has a lower melting temperature (Tm) than IpaD_318, with Tm values of 67.6°C and 69.3°C, respectively [Fig. 6].
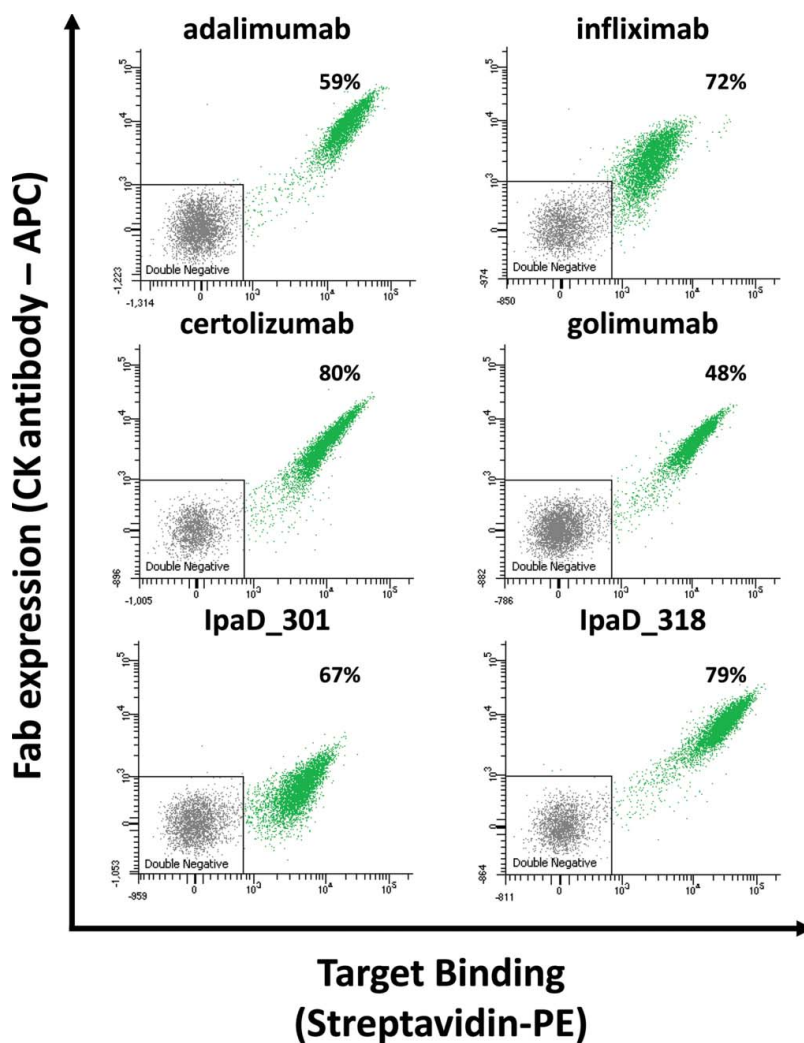
Figure 5. Representative bivariate flow cytometric analysis of yeast cells expressing Fab antibody fragments expressed on their surface. Each single dot on the plot designates the APC (y-axis) and PE (x-axis) fluorescence intensity values for a single yeast cell. Cells were double-labeled with biotinylated antigen/Streptavidin–PE (target binding) and anti-CK APC labels (Fab expression). Antigen concentrations in labeling experiments were 20 nM for anti-TNF antibodies and 50 nM for anti-IpaD antibodies. Functional subpopulations were gated using an inverted gate of the double negative sub-populations. The percentage number correspond to the mean percentage of cells expressing functional Fabs from three separate inductions.
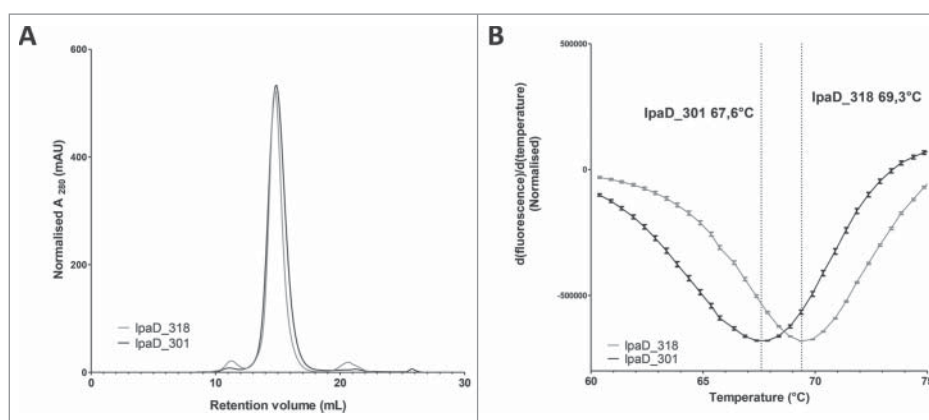
Figure 6. Biophysical characterization of IpaD_301 IgG and IpaD_318 IgG expressed by HEK cells. (A): Size-exclusion chromatograms of the protein A purified IgGs on a Superdex 200 10/300 GL size-exclusion column for IpaD_301 IgG (black) and IpaD_318 IgG (grey). Monomeric IgG species elutes at 15 mL and aggregates or multimeric species elutes between 10 to 12 mL. (B): Tm determination of IgGs by differential scanning fluorimetry. The fluorescence intensities are normalized to peak values. Error bars correspond to experiment triplicates.

Discussion

We carried out a comparative study of expression formats using 6 different antibodies specific for two different antigens, TNF and IpaD. In our hands, Fab appears to be the most versatile expression format for display of functional antibodies on the surface of yeast cells.

ScFv is currently the most widespread format for antibody engineering. It has indisputable advantages, such as its small size, which is particularly convenient for the cloning and sequencing of libraries. Also, numerous studies have used scFvs successfully to engineer clones with improved properties,[4] including clones derived from adalimumab with improved affinity towards TNF.[8] However, some antibodies display reduced affinity and impaired biophysical properties when expressed as scFv as compared to the corresponding Fab molecules.[23,30] Indeed, CH1 and CL domains, which are not present in scFv molecules, participate in the greater stability of Fab and scFab.[31,32] The choice of an adapted format has also proved to be an important parameter for other approaches such as phage display[33,34] or recombinant expression of antibody fragments.[35]

Here, we show that some antibodies do not seem to be active either in the scFv format or in the scFab format when they are expressed at the surface of yeast cells. In our set-up, two antibodies, infliximab and IpaD_301, appeared to lack functionality, as suggested by the total absence of antigen binding in any single-chain format. The presence of C_K and CH1 domains, which could have a stabilizing role in the scFab formats, had no effect on the functionality of the corresponding infliximab and IpaD_301 constructs. We cannot exclude that the presence of a linker between the light chain and the heavy chain could also cause steric hindrance that impairs functionality.

We found that adalimumab, golimumab and IpaD_318 were well expressed and perfectly able to bind their antigen as scFv constructs, but also in scFab or Fab formats. Certolizumab was also a very well expressed and functional antibody in these formats, with the notable exception of the sc60Fab format, which exhibited very poor expression and had no detectable antigen binding (Supp. Fig 2). These particular results emphasize that the presence of the two C-terminal cysteine residues in scFab, as well as the nature and length of the linker sequence, can

dramatically alter expression of scFab molecules for some antibodies. Moreover, the presence of the interchain disulfide bond can have a major influence on the stability of the antibody fragment, as recently shown for the adalimumab Fab.[36] The length of the linker also proved to be an important parameter for the proper packing of light and heavy chains and possibly the accessibility of the paratope, thus ultimately affecting the function of the scFab fragment.[23,24]

In this study set-up, Fab was the only format that allowed functional display for each of the 6 tested antibodies. FACS analysis revealed that a very high proportion of induced yeast cells (up to 70%) expressed Fabs and produced a strong fluorescence signal corresponding to antigen binding. In the context of protein engineering or affinity maturation, a strong signal would favor an easy discrimination of high-affinity clones in libraries containing very high numbers of mutants.

Moreover, for anti-TNF molecules, all Fabs expressed on the surface of yeast cells exhibited a high affinity for their antigen, with K_D values close to those reported in the literature, strongly suggesting that the K_D of a protein-protein binding interaction measured on the surface of yeast cells was essentially equal to that measured using soluble proteins.[37,38]

Deeper analysis of expression reporters in the Fab format indicated a lower display level of light chains for infliximab and IpaD_301 Fabs. Light chain display correlated perfectly with lower target binding of these two constructs compared to the other anti-TNF Fabs or IpaD_318 Fab, respectively. Coexpression of chaperones such as BiP, use of alternative promoters such as *Gal10* for the light chain expression, or introduction of a new secretory leader sequence optimized for antibody secretion such as αMFpp8[39] might be useful to promote the assembly of light and heavy chains of antibodies during Fab expression. A recent study demonstrated that co-expression of protein disulfide isomerase (PDI) had a positive effect on the functionality of some antibodies expressed as Fab on the surface of yeast cells.[40] In this study, Georgiou and co-workers also showed that pairing of antibody chains could be promoted by fusing the light and heavy chains with dimerization domains such as leucine-zipper proteins. Forced-dimerization of antibody chains restored functionality for 3 of 13 tested anti-HA

antibodies, although with a lower proportion of cells displaying functional Fab.

Overall, infliximab and IpaD_301 exhibited relatively low expression in every tested format of antibody fragment. We observed, however, marked differences in the expression profiles of IpaD_301 and IpaD_318 on the surface of yeast cells in spite of their high sequence homology, with only 7 and 8 mutations in the heavy chain and the light chain, respectively. Production of these two antibodies in a full-length IgG format reveal that IpaD_301 has lower production titers in HEK cells and also a lower Tm (67.6°C vs 69.3°C). Interestingly, infliximab is also described as difficult to express in HEK cells[41] and CHO cells,[42] with relatively low stability over time.[43] A comprehensive study comparing the biophysical properties of clinical-stage antibodies indicated that infliximab has much lower production titers in HEK cells than adalimumab, certolizumab and golimumab (6 mg/L for infliximab vs more than 100 mg/mL for the three other anti-TNF antibodies).[41] Wittrup and co-workers also reported that infliximab had a lower Tm (64.5°C vs at least 70°C for the three other anti-TNF antibodies). These observations are consistent with previously published studies indicating that protein secretion by yeast cells is correlated with thermodynamic stability.[44,45] Together, this suggests that antibodies with low thermodynamic stability might have low expression on the surface of yeast cells.

Even as many alternative antibody formats are emerging, most of current therapeutic antibodies on the market are antibodies in the IgG format.[21] Thus, it is essential that the conversion between the screening format – the antibody fragment – and the IgG antibody does not impair the desired properties, and in particular affinity for the antigen. From this perspective, use of Fab has strong advantages. Several reports show that the Fab format tends to retain its binding affinity better than scFv when converted into IgG.[19] Moreover, the presence of a peptide linker in the scFv and scFab formats or introduction of additional domains such as leucine-zipper dimerization proteins might introduce bias in the screening of improved clones because of subtle structural changes. Indeed, some affinity-enhanced molecules engineered in scFv and scFabΔC formats lost affinity after conversion into IgG.[22]

In conclusion, in spite of their practical convenience, single-chain antibody fragment formats such as scFv and scFab do not always lead to a functional display of antibodies at the surface of yeast cells. In contrast, we showed that all 6 tested antibodies were functional in the Fab format, with high affinities for their antigen. Using our Fab expression plasmid, high proportions of yeast cells expressed Fab molecules and exhibited strong antigen binding signals in FACS, two important properties when getting started with the affinity maturation process by yeast display in good conditions. These advantages and the lower risk of losing engineered properties upon conversion into IgG suggest that the Fab format is the best alternative for the engineering of antibodies using YSD.

Material and methods

Yeast display vectors construction

Cloning were performed with the SLiCE recombination cloning method[46] or classic restriction/ligature method using *E.coli*

DH5α strain (Invitrogen). For each anti-TNF antibody, optimized synthetic genes (VH and VL) were ordered from Eurofins Genomics based on the IMGT sequences. All expression plasmids are derived from previously described pCT-L7.5.1[26] and share the *CEN/ARS* replication origin, *TRP* auxotrophy marker, *colE1* replication origin, Ampicillin resistance gene and galactose inducible promoter *GAL1*. Plasmid pCT-L7.5.1 was a gift from Prof. K. Dane Wittrup (Addgene plasmid # 42900).

Plasmids pNT scFv were modified from parental pCT-L7.5.1 plasmid by modifying the aga2p signal sequence with a dipeptide spacer[27] and a *NheI* restriction site. A stop codon was inserted at the 3′ end of aga2p gene to obtain plasmids allowing the display of scFv with a free N-terminus end and a C-terminal anchoring to the yeast cell wall. Synthetic genes corresponding to each scFv were then cloned using *NheI* and *SpeI* restriction sites.

The pNT scFabΔC vector was generated by cloning the coding sequence of the scFabΔC of the previously described pYDscFab[2] between the restriction sites *NheI* and *XhoI* of the pNT scFv plasmid. Plasmid pYDscFab was a gift from Prof. Dennis Burton. Heavy chains and light chains of antibodies were cloned between *NheI/Pfl23II* and *NcoI/SalI* restrictions sites, respectively.

The pNT sc60Fab vectors were generated from pNT scFab plasmids. The linker and the cysteine residues allowing interchain disulfide bond formation were modified by PCR and homologous recombination between *BssHII* and *NcoI* sites. Heavy chains and light chains of antibodies were cloned between *NheI-Pfl23II* and *NcoI-SalI* restriction sites, respectively.

The bigenic pNT Fab vector was generated by ligation into pNT scFv between *SpeI* and *XhoI* of a synthetic gene (Eurofins Genomics) containing a 6-HIS tag, the Aga2p domain, a *Gal1* promoter, an aga2p signal sequence, an HA tag and a transcription terminator. The resulting plasmid has two distinct expression cassettes corresponding to respective expression of light and heavy chains of the Fabs. Heavy chains and light chains of antibodies were then cloned between *NheI/Pfl23II* and *NcoI/SalI* restrictions sites, respectively.

Yeast transformation

Preparation of competent yeast cells EBY100 (ATCC® MYA-4941™; a *GAL1-AGA1:: URA3 ura3-52 trp1 leu2 Δ1 his3 Δ200 pep4:: HIS2 prb1Δ1.6R can1 GAL*) was performed according to Suga et al.[47] Then, 100 μL of EBY100 electro-competent cells were mixed with 1–2 μg plasmid DNA, transferred to a pre-chilled electroporation cuvette (Biorad, 165–2086) and pulsed at 2.5 kV, 25 μF (Biorad Gene Pulser Xcell). Yeast cells were then diluted in 1 mL of sorbitol (1 M), and 200 μL were streaked on SD-CAA agar plates [6.7 g/L yeast nitrogen base without casamino acids, 20 g/L dextrose, 5 g/L casamino acids, 100 mM sodium phosphate pH 6.0].

Growth and expression conditions

Yeast cultures were performed in 24 deep-well round-bottom plates. Pre-cultures were performed by inoculating 3 mL of SD-CAA medium with one colony from selective agar plate and incubated overnight at 30°C, 200 rpm. The saturated pre-

culture (typically OD_{600} of 8–10) was passaged in order to obtain an initial culture OD_{600} of 0.25-0.50. The culture was grown at 30°C until its OD600 reached 0.5-1.0. Cells were centrifuged and re-suspended in 3 mL of SG-CAA galactose induction medium [6.7 g/L yeast nitrogen base without casamino acids, 20 g/L galactose, 5 g/L casamino acids, 100 mM sodium phosphate, pH 6.0] and induced for 16–36 h at 20°C, 200 rpm.

Yeast labelling and flow cytometry analysis

10^6 cells were taken from the induced culture and washed with 1 mL PBSF (phosphate-buffered saline (PBS), bovine serum albumin (BSA) 0.1%) buffer. Cells were resuspended in 50 μL of the target protein at the desired concentration and incubated at 20°C, 1000 rpm until equilibrium is reached (depending on target protein concentration). Cells were washed with 1 mL of ice-cold PBSF to avoid dissociation and resuspended in ice-cold PBSF containing the appropriate fluorescent reporters: Streptavidin-PE (Thermo Fisher scientific; catalog number S866; 1:100 dilution), Mouse anti-Human Kappa Light Chain Antibody APC conjugate (Thermo Fisher scientific; catalog number MH10515; 1:100 dilution), 6x-His Epitope Tag antibody Dylight-650 conjugate (Thermo Scientific, catalog number MA1-21315-D650; 1:100 dilution) or HA Tag Monoclonal Antibody APC conjugate (1:50 dilution). Cells were incubated on ice in the dark for 15 minutes and analyzed with BD FACS Aria[TM] III cytometer.

Cell-Binding assays for determining the affinity of antibodies for their antigen

Cell binding assays were performed according to Hunter et al.[38] in order to avoid ligand depletion. 10^4 to 10^5 cells were incubated in 50–500 μL of the target protein at the desired concentrations and incubated at 20°C, 1000 rpm long enough to reach equilibrium. Cells were analyzed by FACS for their binding fluorescence as described above. Auto-fluorescence was subtracted for each measure and resulting values were normalized with maximum fluorescence. Data are then fitted with a non-linear regression analysis to obtain the K_D.

Production and purification of IgG IpaD_301 and IpaD_318

IpaD_301 and IpaD_318 antibodies were produced via transient transfection of HEK293 Freestyle[TM] cells (HEK 293FS). HEK 293FS were transfected as previously described by Subedi et al.[48] without the use of Valproic acid using the HEK transient transfection plasmids described by Smith et al.[29]

The day before transfection, cells were sub-cultured in order to be at a density of 2–3.10^6 cells/mL the next day. The day of the transfection, cells were centrifuged 5 min at 100 g and resuspended in fresh FreeStyle 293 Expression medium (Thermo-Fisher) to obtain a density of 2.5.10^6 cells/mL. For a transfection volume of 100 mL, 150 μg of each plasmid (coding for heavy chain and light chain) was added to the cells at a concentration of 0.5 μg/μL. Cells were incubated 5 min at 37°C, 180 rpm, 8% CO_2. 1.8 mL of polyethylenimine (PEI) was added

at a concentration of 0.5 mg/mL to the cells (PEI:DNA ratio = 3:1). Cells were subsequently incubated 24h at 37°C, 180 rpm, 8% CO_2.

A day after transfection, 100 mL of fresh EX-CELL® Serum-Free Medium for HEK 293 (Sigma-Aldrich), supplemented with 6 mM L-Glutamine, was added to the transfected culture. Cells were finally incubated 4 more days at 37°C, 180 rpm, 8% CO_2. Five days after transfection, the supernatant was harvested by centrifuging the cells at 1000g for 15 min at 4°C and kept on ice until purification. The supernatant was purified using a HiTrap[TM] Protein A HP column of 1 mL (GE Healthcare) on an ÄKTA purifier (GE Healthcare) according to the manufacturer's description.

Size-exclusion chromatography

A Superdex-200 10/300 GL (GE Healthcare) column was used on an ÄKTA purifier (GE Healthcare). Runs were monitored with Unicorn[TM] 5.31 software (GE Healthcare). 500 μL of each sample was applied on the column using a flow rate of 0.7 mL/min and PBS as running buffer.

Fab IpaD_301 and IpaD_318 affinity determination

Fab were prepared from full-length IgG using a Pierce® Fab Micro Preparation Kit. The binding affinity of Fab IpaD_301 and IpaD_318 was evaluated by biolayer interferometry using an Octet RED96 instrument (Pall ForteBio). Biotinylated IpaD was loaded onto streptavidin biosensors (ForteBio) at a concentration of 5 μg/mL for 8 min in PBSF (1x PBS, pH 7.4, 0.1% BSA). Fab IpaD_301 and IpaD_318 were then associated with IpaD using 4 different concentrations (20, 10, 5 and 2.5 nM). Duration of association and dissociation steps were 20 min and 120 min. All steps were performed at 20°C. Data were collected and analyzed using Octet Software version 10.0.3 (Pall Forte-Bio). Binding kinetics were fitted using a 1:1 Langmuir-binding model.

Thermostability evaluation

DSF was performed as previously described by Niesen et al.[49] using a StepOne Real-Time PCR System from Applied Biosystems. IgG monomers of IpaD_301 and IpaD_318 purified via SEC were mixed with the SYPRO Orange reagent. Final dye concentration was 5x and final IgG concentration was 150 μg/mL. The temperature gradient was run from 20 to 90 °C with 1 min equilibration at each degree centigrade.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Abbreviations

BSA	bovine serum albumin
DSF	differential scanning fluorimetry
Fab	antigen-binding fragment
MFI	mean fluorescence intensity
PDI	protein disulfide isomerase

PEI   polyethylenimine
scFab   single-chain antigen-binding fragment
scFv   single-chain variable fragment
SEC   size exclusion chromatography
TNF   tumor necrosis factor
YSD   yeast surface display

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