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Therapeutic antibodies: Discovery and development using the ProteOn XPR36 biosensor interaction array system

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ABSTRACT

Therapeutic monoclonal antibodies are becoming a significant and rapidly growing class of therapeutic pharmaceuticals. Their discovery and development requires fast and high-throughput methodologies for screening and selecting appropriate candidate antibodies having high affinity for the target as well as high specificity and low cross-reactivity. This study demonstrates the use of the ProteOn XPR36 protein interaction array system and its novel approach, termed One-Shot Kinetics, for the rapid screening and selection of high-affinity antibodies. This approach allows multiple quantitative protein binding analyses in parallel, providing association, dissociation, and affinity constants for several antibodies or supernatants simultaneously in one experiment. We show that the ProteOn XPR36 system is a valuable tool for use across multiple stages of the therapeutic antibody discovery and development process, enabling efficient and rapid screening after panning, affinity maturation, assay validation, and clone selection.

Therapeutic monoclonal antibodies are becoming a significant and rapidly growing class of therapeutic pharmaceuticals for treating a variety of human diseases such as cancer and immunological disorders [1–3]. Currently, there are more than 20 monoclonal antibodies that have been approved as drugs by the U.S. Food and Drug Administration [4]. This growth can probably be attributed to the powerful method of phage display libraries constructed from various genetic sources. It allows the selection of peptides and proteins, including antibodies (usually expressed as scFv and Fab antibody fragments), with high affinity and specificity for a variety of targets and thereby accelerates the drug discovery and development of antibody-based biopharmaceuticals [5–9].

During recent years, it has been well established that human melanoma and other tumor cells express antigens (specific peptides in complex with major histocompatibility complex [MHC][1] class I molecules on the cancer cells) that are recognized by cytotoxic T lymphocytes (CTLs) derived from cancer patients. Therefore, many modern cancer immunotherapy approaches are now designed to induce and enhance T-cell reactivity against these tumor antigens, for example, by adoptive transfer of T cells. However, the major problems with tumor-specific CTLs is that they are relatively rare, there are cases in which the tumor-specific CTLs are either not functional or anergic, and tumors have also developed methods to evade the killing activity of CTLs. Although the use of soluble T-cell receptors (TCRs) seems to be most suitable for this purpose, it is practically difficult because of their inherent low affinity for ligands and their instability as recombinant-engineered molecules. Therefore, a different strategy in which a high-affinity molecule can specifically detect a particular MHC–peptide complex needs to be developed. The most suitable molecules for such an approach can be recombinant antibodies that will have CTL receptor-like specificity mimicking the fine unique specificity of CTLs. Despite many efforts, it has been difficult to generate antibodies with such specificities in the past, but recent advances using phage display technology have overcome these difficulties. Such antibodies, with TCR-like specificity, could serve as good candidates for immunotherapy because they can be used to target cancer cells by constructing recombinant immunotoxins or fusions with cytokine molecules or for bispecific antibody therapy. These antibodies use the same T-cell-based specificity of tumor cell recognition (through tumor-associated MHC–peptide complexes), enabling an antibody-based approach that provides the ability to produce large soluble quantities of potential anti-tumor agents that

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[1] *Abbreviations used:* MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; Ig, immunoglobulin; SPR, surface plasmon resonance; IPTG, isopropyl-β-D-thiogalactopyranoside; cDNA, complementary DNA; RT–PCR, reverse transcription polymerase chain reaction; GLM, general layer medium; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide; sulfo-NHS, sulfo-*N*-hydroxysuccinimide; RU, resonance units; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity.

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penetrate tumors better and exert biologic activity similar to that of T cells [10–13].

Efficient discovery and development of recombinant antibody anti-tumor agents requires rapid generation, screening, and affinity maturation of high-affinity Fab fragments, subcloning to produce intact human immunoglobulin G1 (IgG1) antibodies, and selection of the antibodies with the highest affinity for the TCR antigen as well as affinity measurements of the binding of antibodies to Fc receptors so as to predict effector functions. Tools are needed to expedite and improve the efficacy of all these stages of discovery and development.

Optical biosensors, particularly surface plasmon resonance (SPR)-based instruments, have recently become widely used during the drug discovery and development process. These biosensors can give detailed and valuable information on the binding affinity and kinetics of an interaction without the need for a molecular tag or label. Many of these systems have introduced increased sample throughput with different approaches for sample delivery [14–16]. During the drug discovery and development process, these instruments are of high value and are in use in many stages, including target characterization, compound screening for hits, assay validation, and small molecule detection. They are also used in the development of therapeutic antibodies for screening high-affinity binders, for measuring binding kinetics, and for specificity and concentration assays [17–22].

The ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories, Hercules, CA, USA) can be used for the rapid screening and selection of those antibody binding proteins that provide the optimal binding characteristics for a particular application. It has a unique approach, termed One-Shot Kinetics, that allows multiple quantitative protein binding analyses in parallel, providing association, dissociation, and affinity constants for several antibody binding proteins simultaneously in one experiment [23–25]. The system integrates a unique 6×6 interaction array for the analysis of up to six ligands with up to six analytes, producing 36 data points in a single experiment. Multiplexing improves and expands the capabilities of traditional technology and workflow by enabling multiple quantitative protein binding experiments in parallel. Because multiple conditions can be tested in parallel, comprehensive kinetic analysis of an antibody concentration series can be handled in one experiment. This one-shot parallel approach generates a complete kinetic profile of an antibody interaction without the need for regeneration, in one experiment, using a single sensor chip.

Here we demonstrate the efficient and rapid screening, ranking, and kinetic binding analysis of hundreds of Fab supernatants across multiple stages of the therapeutic antibody discovery and development process, enabling screening after panning, affinity maturation, assay validation, and clone selection. Using the One-Shot Kinetics approach, we were able to screen and determine the kinetic binding constants of hundreds of clones per day.

Materials and methods

Growth and induction of soluble Fab in a 96-well plate

An overnight 96-well plate starter culture of Fab-specific clones was grown at 30 °C. Cells were diluted 1:100 into a new 96-well plate containing 150 μl/well of 2YT/A/0.1% glucose. Clones were grown at 37 °C until OD_{600nm} = 0.9 was reached and then were induced to express the recombinant soluble Fab antibody by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 16 h. The induced 96-well plate was spun at 1700 rpm for 10 min, and supernatants were used for kinetic binding analysis.

Transfection of CHO cells with IgH and IgL expression vectors and screening of cell culture supernatants of clones expressing human IgG1 using SPR analysis

The heavy and light Fab genes were cloned for expression as human IgG1 kappa antibody into mammalian backbone of the eukaryotic expression vector pCMV/myc/ER. For the heavy chain, the multiple cloning sites, the myc epitope tag, and the ER retention signal of pCMV/myc/ER were replaced by a cloning site containing recognition sites for *Bss*HI and *Nhe*I, followed by the human IgG1 constant heavy chain region complementary DNA (cDNA) isolated by reverse transcription polymerase chain reaction (RT–PCR) from human lymphocyte total RNA. A similar construct was generated for the light chain. Each shuttle expression vector carries a different antibiotics resistance gene. Cotransfections of CHO cells with pCMV–IgH and pCMV–IgL expression vectors were performed using the nonliposomal transfection reagent FuGENE 6 (Roche, Belgium). Briefly, 10^6 cells were seeded into 6-well plates, and 24 h after transfection approximately 10 cells/well were placed into 96-well plates in medium containing 1.2 mg/ml G418 and 200 μg/ml hygromycin. Supernatants of single colonies that were grown to near confluence on medium containing selection markers were tested for IgG1 secretion by the ProteOn XPR36 protein interaction array system. The TCRL whole antibody molecules were recovered and purified from culture supernatants by affinity chromatography on protein A columns.

Initial screening for high-affinity Fab fragments and affinity maturation

Immobilization of Fab goat anti-human IgG Fab specific (Jackson ImmunoResearch, West Grove, PA, USA) was performed on a general layer medium (GLM) chip (Bio-Rad Laboratories) at 25 °C in the vertical orientation, and the continuous running buffer was PBST (10 mM Na–phosphate, 150 mM NaCl, and 0.005% Tween 20, pH 7.4). Six channels were activated with 50 μl of a mixture of 0.04 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.01 M sulfo-*N*-hydroxysuccinimide (sulfo-NHS) at a flow rate of 30 μl/min. The Fab anti-Fab was diluted in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 25 μg/ml, and 150 μl was injected followed by an injection of 150 μl of 1 M ethanolamine–HCl (pH 8.5). The immobilization level was approximately 4000 resonance units (RU). Next, 150 μl of five different supernatants was injected in the vertical orientation in five different channels to allow their capture by the immobilized Fab anti-Fab. The sixth channel remained empty to serve as a reference. The MHC I/Tyr antigen (MW = 50 kDa) was injected (75 μl at 50 μl/min) in the horizontal orientation of the ProteOn XPR36 system using five different concentrations (1000, 500, 250, 125, and 62.5 nM). Running buffer was injected simultaneously in the sixth channel for double referencing to correct for loss of the captured supernatant from the chip sensor surface during the experiment. Eventually, the Fab–antigen complex was removed from the immobilized Fab anti-Fab by injecting 50 μl of a regeneration solution of 10 mM glycine–HCl (pH 1.5) in the vertical position. This allowed the capture of five new Fab supernatants.

Data validation of Fab1 binding its target antigen

Immobilization of Fab anti-Fab was performed as described above. The Fab1 supernatant was diluted 2-fold with the running buffer (PBST), and 150 μl was injected in the vertical orientation at a flow rate of 30 μl/min. In this manner, 200 RU of the Fab1 was captured by the immobilized Fab anti-Fab. In a different channel, the purified Fab1 was captured by injecting 150 μl of 10 μg/ml. The immobilization level was 500 RU. The MHC I/Tyr antigen was

injected (75 µl at 50 µl/min) in the horizontal orientation of the ProteOn XPR36 system using five different concentrations (1000, 500, 250, 125, and 62.5 nM).

The purified Fab1 was also used as analyte, and in this case the MHC I/Tyr antigen was immobilized to the chip surface. A mixture of 50 µl of 0.04 EDC and 0.01 M sulfo-NHS at a flow rate of 30 µl/min was injected, followed by an injection of 150 µl of 50 µg/ml neutravidin in sodium acetate buffer (pH 4.5). Deactivation was performed by injecting 150 µl of 1 M ethanolamine–HCl (pH 8.5). The biotinylated MHC I/Tyr antigen was captured by injecting 150 µl of 10 µg/ml. Approximately 1500 RU was captured. Next, the purified Fab1 was injected as analyte (75 µl at 50 µl/min) in the horizontal orientation of the ProteOn XPR36 system using five different concentrations (1000, 500, 250, 125, and 62.5 nM).

Expression levels and binding analysis of the entire full human IgG antibody

Immobilization of protein A was performed on a GLM chip (Bio-Rad Laboratories) at 25 °C in the vertical orientation, and the continuous running buffer was PBST. Two channels were activated with 50 µl of a mixture of EDC and 0.01 M sulfo-NHS at a flow rate of 30 µl/min. Protein A was diluted in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 25 µg/ml, and 150 µl was injected followed by an injection of 150 µl of ethanolamine to deactivate any remaining activated carboxylic groups. The immobilization level was approximately 4000 RU. Next, 150 µl (at 30 µl/min) of five different IgG supernatants was injected in the horizontal orientation in five different channels to allow their capture by the immobilized protein A. Running buffer was injected simultaneously in the sixth channel for double referencing. A separate injection comprising five different human IgG standard concentrations (2.5, 1.5, 1.0, 0.5, and 0.1 µg/ml) was performed for qualitative assessment of the IgG concentrations and, hence, the expression levels. The MHC I/Tyr antigen and a nonrelevant antigen (MHC I/Mart) were injected (60 µl at 30 µl/min) in the vertical orientation (in channels 1 and 2, respectively) of the ProteOn XPR36 system at a single concentration of 1000 nM. Eventually, the IgG–antigen complex was removed from the immobilized protein A by injecting 50 µl of a regeneration solution of 10 mM glycine–HCl (pH 2.0) in the vertical position. This allowed the capture of five new IgG supernatants for further cycles.

Comparing the binding kinetics of different Fab1 constructs

For the IgG antibody measurement, protein A was immobilized on a GLM chip as described above. The IgG form of Fab1 was diluted in PBST to 5 µg/ml, and 90 µl was injected in the vertical orientation at a flow rate of 30 µl/min. For the Fab measurements, Fab anti-Fab was immobilized as described above. The Fab1 was diluted to 10 µg/ml in PBST, and 150 µl was injected at 30 µl/min. Five concentrations of the MHC I/Tyr antigen (1000, 500, 250, 125, and 62.5 nM) were then injected (75 µl at 50 µl/min) in the horizontal orientation. Running buffer was injected in the sixth channel.

For the scFv form of Fab1, neutravidin (Pierce, Rockford, IL, USA) was immobilized by activating the surface with 50 µl of a mixture of 0.04 M EDC and 0.01 M sulfo-NHS at a flow rate of 30 µl/min. Neutravidin was diluted in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 50 µg/ml, and 150 µl was injected followed by an injection of 150 µl ethanolamine to deactivate any remaining activated carboxylic groups. The immobilization level was approximately 9000 RU. The biotinylated MHC I/Tyr was diluted in PBST to 10 µg/ml, and 150 µl was injected in the vertical orientation. Five concentrations of the scFv (1000, 500, 250, 125, and 62.5 nM) were injected as analyte (75 µl) in the horizontal orientation at a flow rate of 50 µl/min. Running buffer was injected in the sixth channel.

Fc gamma receptor interaction with target IgG antibody

The wild-type IgG and two Fc variants (double and triple mutants at the Fc: S239D/I332E and S239D/I332E/A330L) were immobilized on a GLM chip (Bio-Rad Laboratories) at 25 °C in the vertical orientation, and the continuous running buffer was PBST. Three channels were activated with 30 µl of a mixture of 0.02 EDC and 0.005 M sulfo-NHS at a flow rate of 60 µl/min. The three IgG antibodies were diluted in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 25 µg/ml, and 120 µl was injected followed by an injection of 150 µl of ethanolamine to deactivate any remaining activated carboxylic groups. Six different concentrations (80, 40, 20, 10, 5, and 2.5 nM) of the Fc γ RIIIa (CD16a) antigen (R&D Systems, Minneapolis, MN, USA) were injected (30 µl at 60 µl/min) simultaneously as analyte in the horizontal orientation of the ProteOn XPR36 system. Regeneration was performed with 10 mM glycine–HCl (pH 2.5).

Data processing and analysis

All binding sensorgrams were collected, processed, and analyzed using the integrated ProteOn Manager software (Bio-Rad Laboratories). Binding curves were fitted using the Langmuir model describing 1:1 binding stoichiometry or with the Langmuir and mass transfer limitation model. Each individually captured antibody interacting with the five concentrations of antigen was fitted using a global k_a, k_d, and R_{max}. Global fitting is used when the same k_a, k_d, and R_{max} values describe a specific biological model such as five antigen concentrations interacting with a certain antibody. k_a is the association rate constant for the antibody–antigen binding, k_d is the dissociation rate constant for the pair, and R_{max} describes the signal (in RU) when all of the ligand binding sites are saturated by the analyte.

Results

The entire study and workflow aimed at identifying and isolating a Fab fragment having high affinity to the target antigen MHC I/Tyr complex. This complex is composed of a Tyr peptide that is a tyrosinase-derived peptide associated with class I molecules of the MHC and acts as an antigen for the immunological recognition of melanoma. The study involved numerous binding assays, all of which were performed using the ProteOn XPR36 protein interaction array system. These included screening assays, affinity ranking, and kinetic binding analysis of hundreds of Fab supernatants. Kinetic binding and affinity constants of the different Fab supernatants interacting with the antigen were determined using the special assay configuration and the parallel data collection mode of the ProteOn XPR36 system. This configuration, optimized for rapid and efficient binding analysis, allowed the full determination of kinetic binding constants of five different Fab supernatants in each single cycle. The general assay configuration was as follows (Fig. 1):

1. Fab goat anti-human Fab was immobilized covalently on six channels using amine coupling chemistry in the vertical orientation.
2. Five different Fab supernatants were captured by the immobilized Fab goat anti-human Fab in the vertical orientation.
3. Five different concentrations of the antigen were injected simultaneously in the horizontal orientation. Running buffer was injected in the sixth channel as a reference (double referencing) to correct for the decay of the Fab supernatant from the Fab goat anti-human Fab capture agent.

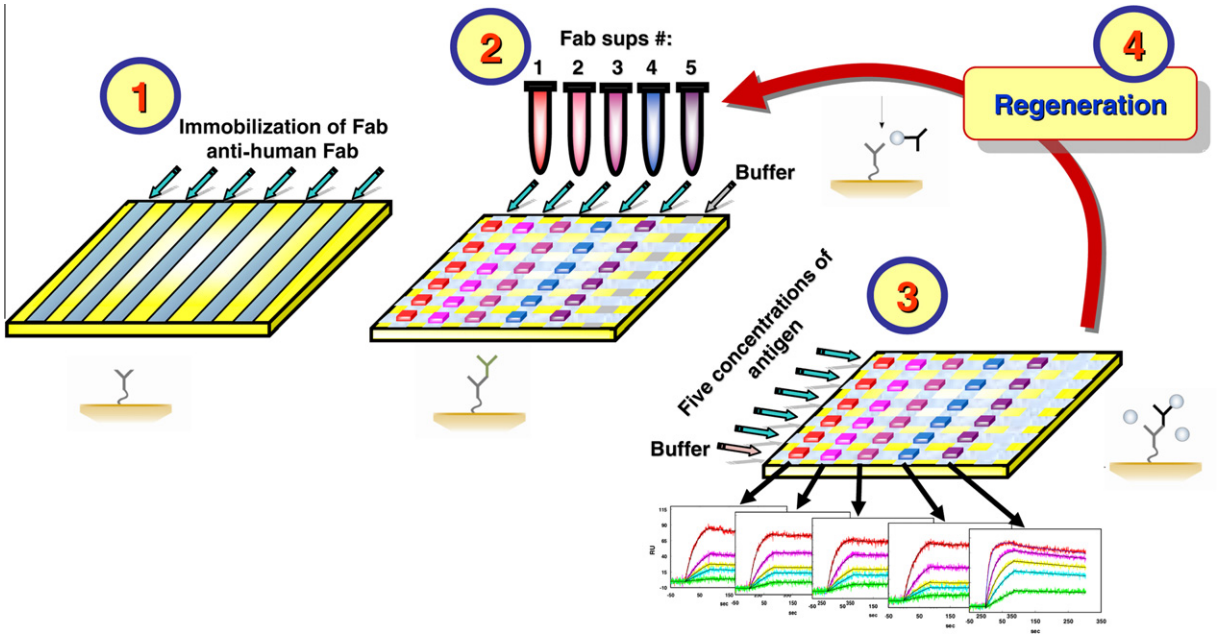

Fig. 1. Workflow for the kinetic analysis of supernatant antibodies. The steps are as follows: (1) Fab goat anti-human IgG Fab specific is covalently immobilized in six vertical channels; (2) five different Fab supernatants (sups) are injected in the vertical channels; (3) different concentrations of the antigen and running buffer are injected in the six horizontal channels, and kinetic data are collected; (4) the antigen/Fab complex is stripped from the sensor chip, which is regenerated to allow the capture of five new supernatant antibodies.

4. A regeneration step was performed to remove the captured Fab fragments, allowing the capture of five different additional Fab supernatants.

Initial screening for high-affinity Fab fragments

Following panning and enrichment steps, the different clones were screened to determine their binding kinetic and affinity constants using the ProteOn XPR36 system. A Fab anti-Fab was immobilized on a GLM chip using standard amine coupling. Five different clones were then captured by the Fab anti-Fab capture agent. Finally, five different concentrations of the antigen were injected in parallel, with running buffer being injected in the sixth channel for double referencing. After data collection, the surface was regenerated to remove the Fab supernatants–antigen complex so as to allow the capture of five new Fab supernatants. In this manner, the interactions of 62 different Fab supernatants interacting with the antigen were monitored in 13 cycles (Fig. 2). All of the binding curves were fitted using the Langmuir model describing a 1:1 binding stoichiometry. For each Fab supernatant interacting with the five antigen concentrations, single global k_a, k_d, and R_{max} were fitted. The kinetic binding constants and the affinity constants (from the relation of $K_D = k_d/k_a$) were determined and plotted in a k_a versus k_d graph (Fig. 3). Most supernatants have affinity constants of approximately 1 μM. Two supernatants, however, showed much stronger affinities in the range of 5 nM (Fab1) and 80 nM (Fab2).

Data validation of Fab1 binding its target antigen

To validate these results, we measured and compared the binding constants for the interaction of the Fab1 clone with its target antigen using different approaches: (i) supernatant of Fab1 was captured by a Fab anti-Fab, and the target antigen was used as analyte; (ii) purified Fab1 was captured by a Fab anti-Fab, and the target antigen was used as analyte; (iii) the antigen was immobilized to the chip surface, and the purified Fab1 was used as analyte. The binding curves are shown in Fig. 4, and the kinetic constants are summarized in Table 1. The results show that the kinetic binding constants are similar for all three cases even when the reaction was reversed, with the antigen being immobilized and the Fab being injected as analyte. Because the Fab fragment is capable of binding only a single antigen molecule (monovalent), it is possible to compare the kinetic binding constants when the assay is performed with the antigen serving as either analyte or ligand. These results served to verify and validate the initial observations during the initial screening assays.

Affinity maturation of Fab1 and Fab2

The native Fab1 clone having an affinity of 5 nM was further subjected to affinity maturation by VL shuffling to increase its affinity. Fab mutants originating from Fab1 were then screened to determine their kinetic binding constants and affinities toward the target antigen. The screening assay configuration was performed similar to the initial screening described in Fig. 1. Thus, five different Fabs were captured in a single cycle, and then five different concentrations of the target antigen were injected in parallel. In this manner, 40 mutant Fab supernatants were screened in a total of eight cycles. The binding curves of representative mutants are shown in Fig. 5A. In this figure, it can be seen that a variety of kinetic profiles exist for the different Fab1 mutants. The k_a and k_d values were plotted in a k_a versus k_d plot shown in Fig. 6. From this plot, it is evident that the mutations had a greater effect on the dissociation rate constants and a lesser effect on the association rate constants. Affinity constants of the various mutants ranged from 100 nM to low nanomolar values.

Similarly, the native Fab2 clone having an affinity of 80 nM was also subjected to affinity maturation. As for the Fab1 clone, the mutations affected mostly the dissociation rate and the obtained affinities spanned from less than 1 μM to low nanomolar values. Approximately 260 different supernatants were tested in 52 cycles. Representative binding curves are shown in Figs. 5B, and the k_a versus k_d plot is shown in Fig. 6.

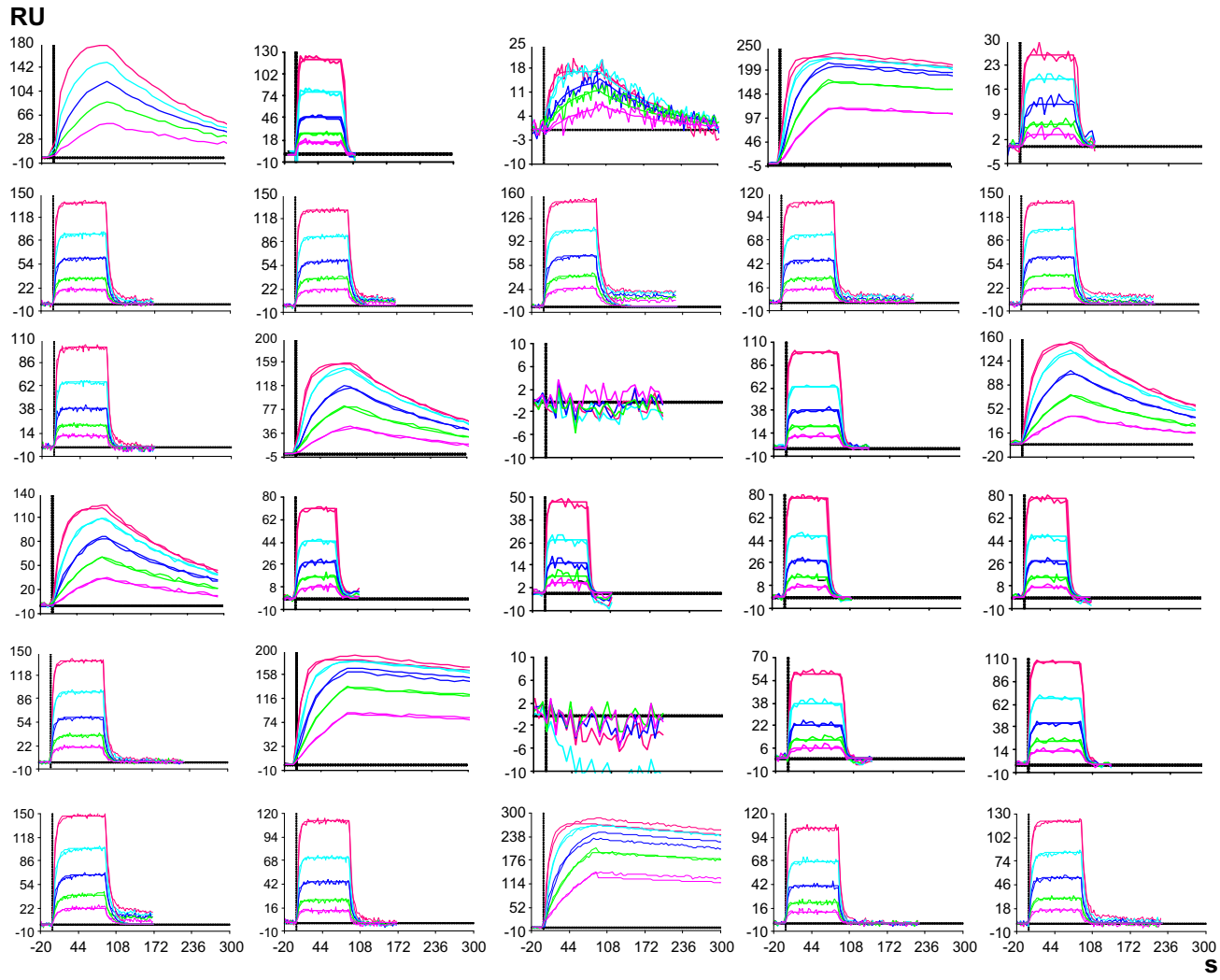

Fig. 2. Representative binding curves from the initial screening assay of Fab fragments interacting with the target antigen.

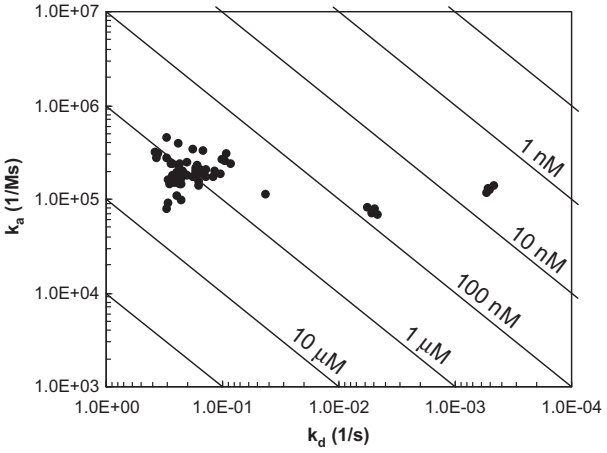

Fig. 3. Kinetic binding constants plotted on a k_a versus k_d plot for the interactions of the different Fabs with the antigen. Data points along the same diagonal have identical binding affinities.

Interestingly, the affinity maturation process was more efficient for Fab2 generating mutants with improved affinities in the low nanomolar range (K_D = 80 nM for the wild-type Fab2). The process was less efficient for Fab1 given that no significant improvement in the affinity constant (K_D) was obtained.

From a Fab fragment to an entire full human IgG antibody

The selected Fab fragments (Fab1 and Fab2) were individually subcloned to form an entire full human IgG1 antibody. The DNA vectors encoding for the heavy and light chains of the whole IgG1 were cotransfected to CHO cells. Following transfection, a clonal selection was performed and the expression level, affinity, and specificity of the different IgG clones for the target antigen were tested using the ProteOn XPR36 system. First, protein A was covalently immobilized using amine coupling chemistry in two channels in the vertical orientation to allow the capture of the expressed IgG antibodies. Second, five different IgG supernatants containing the antibodies were injected in five channels in the horizontal orientation to be captured by the immobilized protein A capturing agent (Fig. 7, left panels). For qualitative measurement of the different IgG clone expression levels, a separate injection comprising five different human IgG standard concentrations (2.5, 1.5, 1.0, 0.5, and 0.1 µg/ml) was performed (red curves in Fig. 7). The binding levels of the different IgG supernatants could be compared with those of the standard concentrations and thereby facilitate the selection of high-expression-level clones.

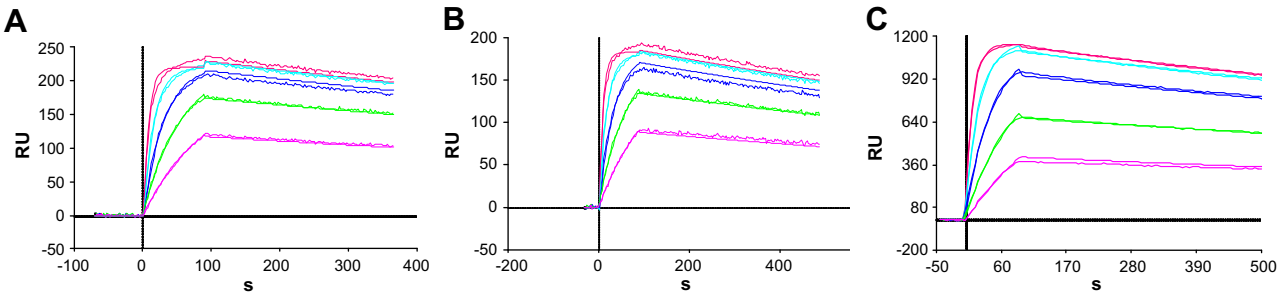

Fig. 4. Binding analysis of the Fab1 clone using three different approaches. (A) Supernatant of Fab1 was captured by a Fab anti-Fab, and the target antigen was used as analyte. (B) Purified Fab1 was captured by a Fab anti-Fab, and the target antigen was used as analyte. (C) The antigen was immobilized to the chip surface, and the purified Fab1 was used as analyte.

Table 1
Validation of kinetic binding constants of the Fab1 clone interacting with the MHC I/Tyr target antigen.

Binding configuration	k_a (1/ms)	k_d (1/s)	K_D (M)
Supernatant Fab1 captured by a Fab anti-Fab; antigen used as analyte	1.32×10^5	5.47×10^{-4}	4.15×10^{-9}
Purified Fab1 captured by a Fab anti-Fab; antigen used as analyte	1.21×10^5	5.37×10^{-4}	4.45×10^{-9}
Antigen immobilized to the surface and purified Fab1 as analyte	8.04×10^4	4.22×10^{-4}	5.25×10^{-9}

Third, to verify that the kinetic binding profiles and the affinities of the whole IgG were maintained during the subcloning process, we monitored the interaction of the captured IgG (by protein A) with its target antigen and also with a nonrelevant antigen (negative control). Thus, the antigen and the nonrelevant antigen were injected in channels 1 and 2, respectively, in the vertical orientation immediately after the capture step (Fig. 7, middle and left panels). Binding curves of the interaction between the different IgGs and the specific antigen were qualitatively compared with binding profiles of the original Fab fragments. The results show very similar binding profiles for the subcloned IgG and the original corresponding Fab fragment, indicating that the affinity was not changed during the process of converting the Fab fragment to a whole IgG molecule. In addition, the nonspecific antigen did not interact with any of the IgG molecules, thereby confirming the preservation of the antibody specificity. After data collection that included the capture of IgG by protein A followed by specific and nonspecific antigen interaction, a regeneration step was performed to allow the testing of five additional new IgG antibodies. This assay configuration allows the determination of both IgG expression levels and affinity measurements using a single ProteOn XPR36 chip.

Kinetic binding constants of different Fab1 immunoglobulin constructs

Because the desired antibody can be used for therapy both as an armed fragment such as scFv or Fab immunotoxin and as a naked IgG, it is important to confirm that, on conversion, these constructs maintained their affinity toward the target antigen. Therefore, we measured and compared the binding kinetics and the affinities of the interaction of the MHC I/Tyr antigen with three different Fab1 forms: a whole IgG, a Fab, and an scFv. For the whole IgG antibody and Fab fragment measurements, the MHC I/Tyr antigen was injected as the analyte. However, for the scFv fragment, the antigen was immobilized and the scFv fragment was injected as the analyte. The binding curves of the three constructs interacting with the target antigen are shown in Fig. 8. The results of the detailed kinetic analysis revealed similar kinetic binding constants and affinities, as can be seen in Table 2. This suggests that the binding kinetic profiles of the Fab, scFv, and whole IgG molecule were not altered on conversion.

Interaction of Fc gamma receptor with Ab2 IgG antibody and its Fc mutants

The action and efficiency of antibodies are largely dependent on their antigen binding ability but are also dependent on effector functions such as the ability of the antibodies to recruit immune system components following antigen binding. These effector functions are antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). There are several known mutations in the IgG Fc region that alter the activities of these effector functions [6]. Therefore, we constructed two Fc variants from the wild-type Ab2 IgG (the whole IgG form of Fab2) to include double and triple point mutations. These were subjected to detailed kinetic binding analysis to determine their binding constants for the interaction with an Fc gamma receptor (immobilized on the chip). The binding curves are shown in Fig. 9, and the kinetic binding constants for the interaction of the Fc gamma receptor with Ab2 antibody and its two Fc mutants are shown in Table 3. The affinities of the two mutants toward the Fc gamma receptor increased more than 30-fold compared with the affinity of the Ab2 wild type. Most of the change is probably attributed to the decrease in the dissociation rate. The affinities of the two mutants, however, were very similar.

Discussion

Recombinant antibodies that mimic the fine unique specificity of CTLs could be very useful as potential anti-tumor agents. Many cancer immunotherapy approaches are designed to induce and enhance T-cell reactivity against tumor antigens, for example, by adoptive transfer of T cells. However, tumor-specific CTLs can be rare, nonfunctional, or anergic, and tumors have also developed methods to evade the killing activity of CTLs. Although soluble TCRs seem to be most suitable for this purpose, the inherent low affinity of TCRs for ligands and their instability as recombinant-engineered molecules often render them unsuitable as anti-tumor agents.

Although recombinant antibodies with TCR-like specificity are logically good candidates for cancer immunotherapy, it has been difficult to generate antibodies with such specificities in the past. However, recent advances using phage display technology have overcome these difficulties. These antibodies use the same T-cell-based specificity of tumor cell recognition, penetrating tumors better and exerting biologic activity similar to T cells [10–13] while offering the ability to produce large soluble quantities of the potential anti-tumor agents.

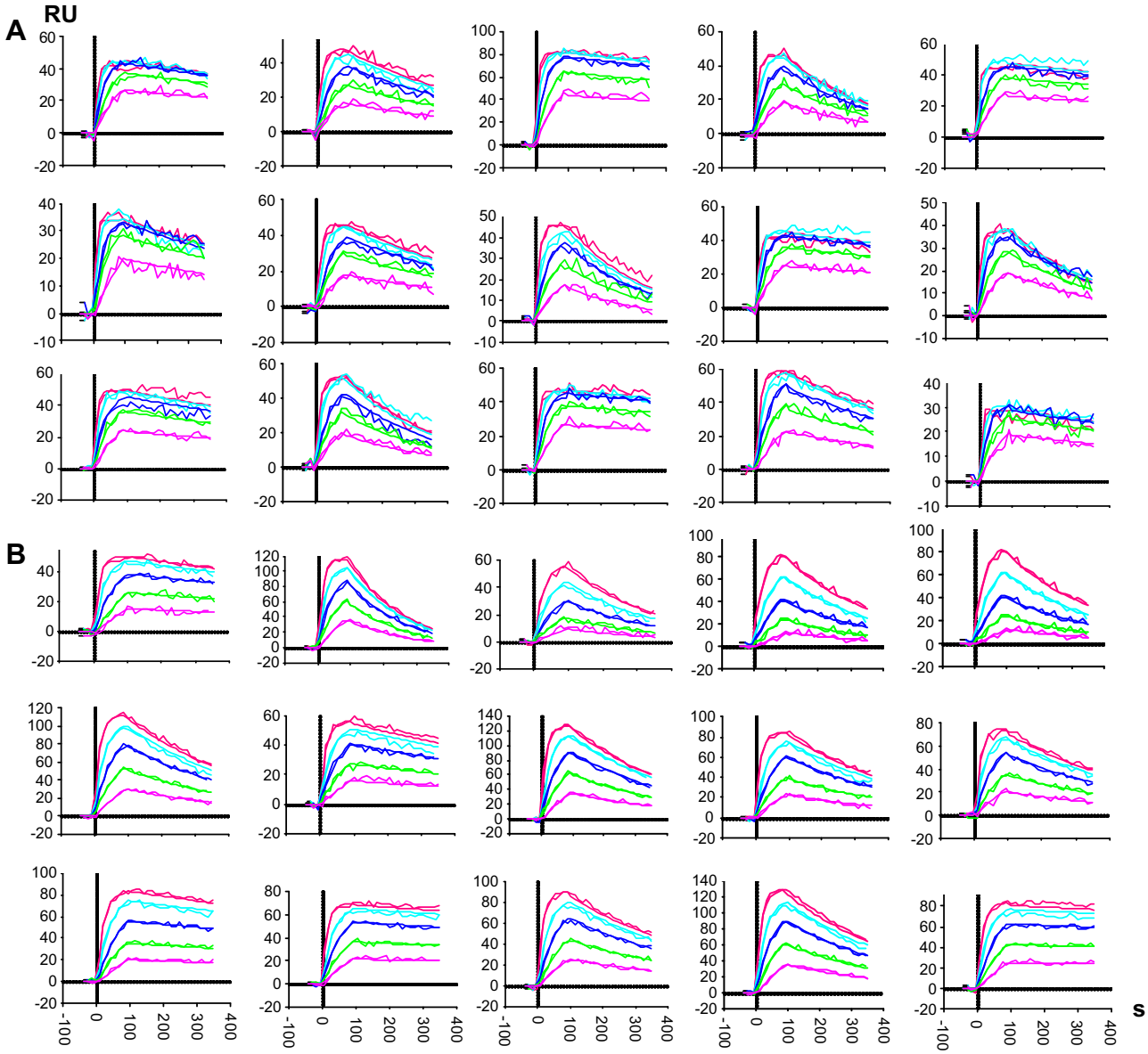

Fig. 5. Binding curves for the screening of affinity maturated fragments: (A) Fab1; (B) Fab2.

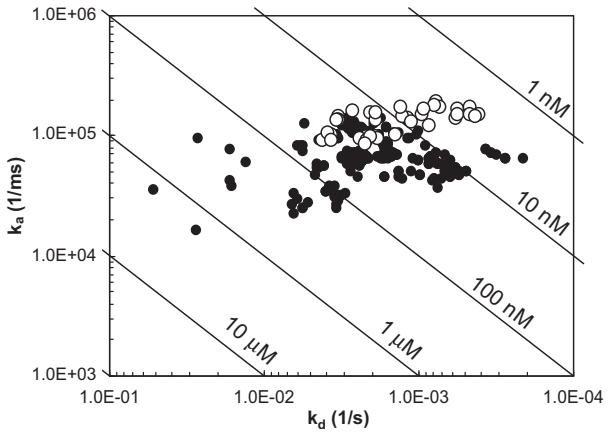

Fig. 6. Kinetic binding constants plotted on a k_a versus k_d plot for the interactions of the different affinity maturated fragments with the antigen. Open circles: Fab1; closed circles: Fab2.

Efficient discovery and development of recombinant antibody anti-tumor agents requires rapid generation, screening, and affinity maturation of high-affinity Fab fragments, subcloning to produce intact human IgG1 antibodies, and selection of the antibodies with the highest affinity for the TCR antigen as well as affinity measurements of the binding of antibodies to Fc receptors so as to predict effector functions. Using a large human antibody phage display library, large numbers of recombinant antibodies that transform the unique fine specificity but low intrinsic affinity of TCRs into high-affinity soluble antibody molecules endowed with a TCR-like specificity toward human tumor epitopes have been developed [12]. Screening of the large number of candidate Fab-specific clones, as well as the capture and analysis of the subsequent and various Fab immunoglobulin constructs, demands a method that can be highly multiplexed while maintaining the ability to discern small differences in affinity for the TCR antigen.

The multiplex advantage of the ProteOn XPR36 protein interaction array system enables the rapid screening of large numbers of monoclonal antibody supernatants to accurately identify high-affinity, high-specificity antibodies for antigens of interest. The ki-

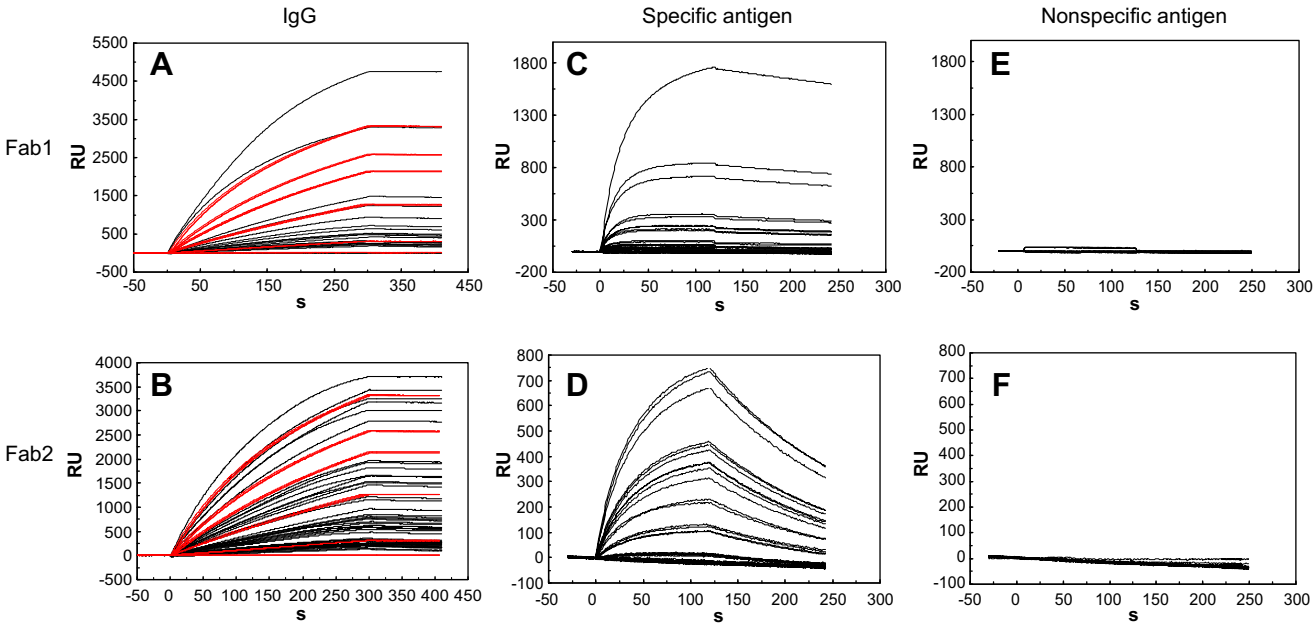

Fig. 7. Whole IgG (IgG forms of Fab1 and Fab2) binding analysis for both expression level measurements of the different clones and kinetic binding profiles for the interactions with a specific and nonspecific antigen. (A) IgG supernatants (IgG form of Fab1) interacting with protein A. (B) IgG supernatants (IgG form of Fab2) interacting with protein A. (C) Interaction of the captured IgG (IgG form of Fab1) by protein A with its target antigen. (D) Interaction of the captured IgG (IgG form of Fab2) by protein A with its target antigen. (E) Interaction of the captured IgG (IgG form of Fab1) by protein A with a nonrelevant antigen. (F) Interaction of the captured IgG (IgG form of Fab2) by protein A with a nonrelevant antigen.

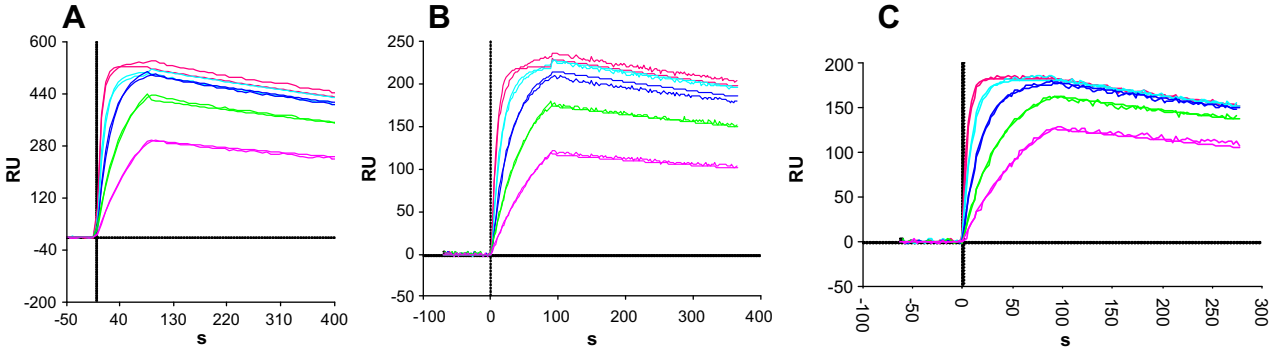

Fig. 8. Binding curves of the antigen interacting with different Fab1 constructs: (A) scFv; (B) Fab; (C) IgG.

Table 2
Kinetic binding constants of different constructs of the Fab1 clone interacting with the MHC I/Tyr antigen.

	k_a (1/ms)	k_d (1/s)	K_D (M)
scFv	1.58×10^5	5.92×10^{-4}	3.73×10^{-9}
Fab	1.32×10^5	5.47×10^{-4}	4.15×10^{-9}
IgG	1.31×10^5	7.77×10^{-4}	5.92×10^{-9}

netic analyses can be performed without the need for antibody purification. Data collection is performed in cycles of up to six different supernatants each, and the binding kinetic constants of the six supernatants in each cycle are determined in parallel. This parallel analysis mode enables correction for the drift (but not for the change in surface capacity) caused by the decay of the capture agent/supernatant antibody complex, thereby improving the accuracy of the analysis. Using this approach, the kinetic analysis of hundreds of different clones raised against the MHC I/Tyr TCR antigen could be completed per day.

Most important, the high throughput of the ProteOn XPR36 system expedites every step of the recombinant antibody discovery and development process. Following affinity maturation of Fab1 and Fab2 clones, 300 mutant Fab supernatants were rapidly screened to select those with the highest affinity. Intact human IgG1 antibodies produced by subcloning of the Fab1 and Fab2 fragments were also screened using the ProteOn XPR36 system to ensure that the kinetic binding profiles were maintained during the subcloning process. Expression level and specificity of each recombinant IgG1 were also determined using the ProteOn XPR36 system. Kinetic studies also suggested that the binding kinetic profiles of the Fab, scFv, and whole IgG molecule were not altered on conversion. Finally, the ProteOn XPR36 system was used to identify Fc variants that increased the affinity for the Fc gamma receptor more than 30-fold, thereby potentially modifying their ADCC and CDC effector functions.

SPR platforms with high-throughput capabilities have been used previously for screening and affinity ranking of hundreds of antibodies during the development of therapeutic antibodies within a relatively short time. For example, screening for high-affinity antibodies in which 400 different Fab fragments can interact with a single antigen concentration in a single injection, allowing their

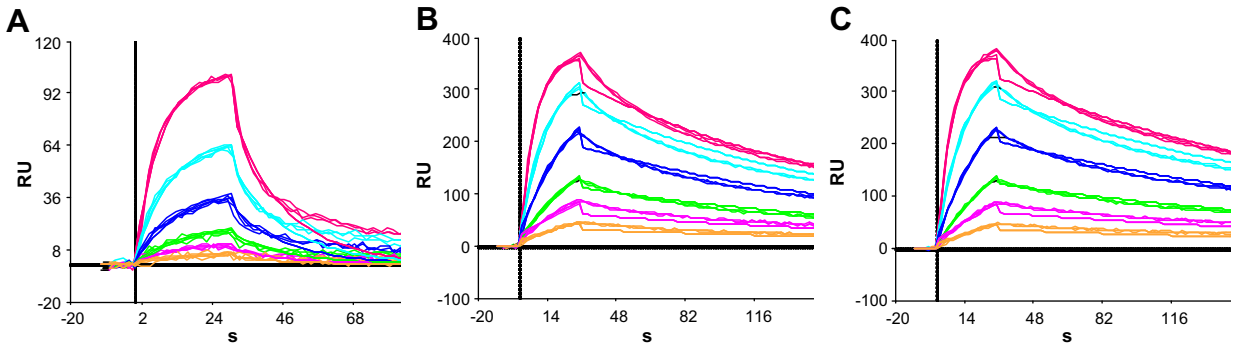

Fig. 9. Binding curves for the interaction of the Fc gamma receptor (immobilized on the chip) with the IgG form of Fab2. (A) Wild-type IgG. (B) Two point mutations in the Fc region. (C) Three point mutations in the Fc region.

Table 3
Kinetic binding constants for the interaction of the Fc gamma receptor with the Fab2 IgG antibody and its Fc mutants.

	k_a (1/ms)	k_d (1/s)	K_D (M)
IgG, wild type	1.17×10^6	8.10×10^{-2}	6.91×10^{-8}
IgG, two mutations	2.17×10^6	7.09×10^{-3}	3.28×10^{-9}
IgG, three mutations	1.99×10^6	5.25×10^{-3}	2.64×10^{-9}

ranking based on their affinity, has been described before [26]. Screening with captured antibodies using Fc-specific antibody surfaces and monitoring antigen binding at a single concentration enabled the characterization of binding kinetics to approximately 200 antibody supernatants per day [27]. Also, Säfsten and coworkers described the screening of 384 supernatant antibodies within 12 h using a single antigen concentration. They reported that although this single-concentration kinetic approach is not ideal, it can be used for ranking purposes [28]. In another work, isolated clones expressed as soluble Fab fragments were screened and ranked based on their affinity against the target, taking advantage of the ability to measure Fab concentrations in crude bacterial extracts [29]. SPR platforms have also been used previously to characterize engineered antibody Fc variants with enhanced effector function [30].

The primary advantage of the ProteOn XPR36 system, however, is that it allows the full kinetic determination of up to six different antibodies simultaneously using six different antigen concentrations in parallel in a single run [15,24], enabling it to screen 250 monoclonal antibody supernatants in approximately 17 h [23]. In addition to its higher throughput, the ProteOn XPR36 system has been shown to readily detect even relatively minor differences in kinetic constants when screening antibody library clones [31]. The current work has shown that the ProteOn XPR36 protein interaction array system is a valuable tool for use across multiple stages of the therapeutic antibody discovery and development process, enabling rapid and efficient screening after panning, affinity maturation, assay validation, and clone selection.

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