Cell lines

Cells were cultured in RPMI-1640 plus GlutaMAX supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate and 1% MEM nonessential amino acids at 37°C and 5% CO_2 in a humidified atmosphere.

FACS Scatchard analysis

For FACS-based Scatchard analysis, CD37-positive Ramos Burkitt lymphoma cells were used as target cell. Serial antibody dilutions were performed in a 96-well plate starting with 160 µL of a 100 nM dilution of antibody, followed by 11 additional dilution steps (1:2; 80 µL antibody + 80 µL PBS/BSA 0.5% + 0.02% sodium azide). 50 µL of each dilution were added to FACS tubes, thereafter 150 µL Ramos cells (0.8×10^6/mL = 1.2×10^5 cells/tube) were added to each tube. Cells were gently mixed and incubated for 1 hour on ice. 50 µL of FITC conjugated secondary antibody (mouse anti-human IgG 15 µg/mL) was added to each tube, mixed, and incubated for 30 minutes on ice. Thereafter 4 mL PBS pH 7.2 (0.02% sodium azide) was added, cells were centrifuged (1000 rpm, for 5 minutes), supernatant was removed and cells were suspended in 300 µL PBS pH 7.2 (0.02% sodium azide). Samples were analyzed on a BD FACS Canto Flow Cytometer using the BD FACS Diva software (Version 5.0.2). For quantification of FITC fluorescence intensity a Quantum™ FITC MESF Premix kit (Bangs Laboratories) was used. The number of FITC molecules per cell was calculated according to the manufacturer's instructions, using a calibration curve obtained by measuring 4 populations of calibrated fluorescent standards provided with the kit. The number of bound antibody molecules per cells was calculated according the following formula: Bound IgG (B) = (Cells/mL * FITC/cell) / (F/P ratio); where F/P ratio = number of fluorescent dye molecules per molecule of secondary antibody. Free IgG (B) = Total IgG – Bound IgG (B). The bound versus free (B/F) antibody ratio was plotted against the number of bound antibody molecules (B) and the slope of the curve (S)

was determined. The affinity of the antibody was determined using the following formula: K_D (M) = 1/ S.

Surface plasmon resonance (SPR)

All kinetic SPR binding studies were performed at 25°C on a Biacore T200 instrument (GE Healthcare) and recorded with a data collection rate of 10 Hz. Each of the several mAb/FcγR interaction experiments was set up in a way that the antibodies were immobilized ligands captured on the surface of a single, reusable Protein A sensor chip, while Fcγ receptors CD16 and CD64 were used as soluble analytes (Fig. S1). We note here that antibodies are engaged by Fcγ receptors in an asymmetric 1:1 mode,[1–3] and that in consequence, avidity effects do not have to be considered in the experimental design. For the Protein A chip preparation, similar amounts of 2,730 and 2,690 resonance units (RU) of recombinant Protein A (GE Healthcare) were covalently immobilized to the upstream (reference) and downstream (experimental) flow cell surface of a CM5 biosensor chip (GE Healthcare), respectively, using standard amine-coupling chemistry (GE Healthcare) following manufacturer's instructions. Antibodies were diluted in HBS-ET running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) Tween 20, pH 7.4 at 25°C) and noncovalenty bound to the experimental flow cell surface at a flow rate of 5 µL/minute. Analyte stock concentrations were calculated by using absorbance values at 280 nm and theoretical extinction coefficients. The analytes were serially diluted in running buffer and injected in series over the reference- and experimental (ie mAb coated) biosensor surface at a flow rate of 50 µL/minute. At the end of each cycle, the biosensor surface was regenerated with two injections (60 and 30 seconds, respectively) of 10 mM glycine pH 1.7 at a flow rate of 5 µL/minute. For each analyte sample injection (ie each cycle), binding responses obtained from the experimental biosensor surface were double referenced by subtracting simultaneously recorded responses from the reference surface followed by additional subtraction of responses from a single referenced running buffer sample. In case of

mAb/CD64 interactions, the association (on-rate) and dissociation (off-rate) constants (k_a and k_d) were determined simultaneously by globally fitting double-referenced sensorgrams of the entire titration series to a “1:1 binding” model (Fig. S2 E, F) using Biacore T200 Evaluation Software 1.0 (GE Healthcare). In this case, the dissociation constant, K_D, was calculated from the determined rate constants by the relation $K_D = k_d/k_a$.

In case of mAb/CD16 interactions the “1:1 binding model” was not suitable and resulted in large deviations between fit and data. Only the “two-state” model, a model that assumes a complex stabilizing confirmational change after an initial complex of ligand and analyte has been formed, resulted in fits of high quality (Fig. S2 A–D). By using a “two state” model to fit the data, four rate constants (k_{a1}, k_{d1}, k_{a2} and k_{d2}) are reported by the Biacore evaluation software. We observed the most significant differences in the k_{a1} and especially k_{d1} rate constants (Table S1) when we compared the binding of different IgG1 antibodies to CD16. In addition, these two rate constants kinetically describe the association and dissociation events of ligand and analyte ($A + B \leftrightarrow AB$) rather than the conformational change of $AB \leftrightarrow AB^*$ (kinetically described by k_{a2} and k_{d2}). These two observations led us to report a K_{D1} value that is calculated by the relation $K_{D1} = k_{d1}/k_{a1}$.

Table S1. Kinetic and affinity data determined by SPR

Experimental setup		Kinetic analysis						
Immobilized ligand	Soluble analyte	k_{a1} [$M^{-1} s^{-1}$]	k_{d1} [s^{-1}]	K_{D1} [nM]	k_{a2} [s^{-1}]	k_{d2} [s^{-1}]	K_{Dtot} [nM]	R_{max} [RU]
mAb 37.1-wt	CD16V	1.57 (±0.02) • 10^6	0.338 (±0.004)	215	0.017 (±0.001)	0.028 (±0.001)	135	27
mAb 37.1		4.12 (±0.01) • 10^6	0.016 (±0.001)	4	0.013 (±0.001)	0.011 (±0.001)	1.8	51
Rituximab		2.11 (±0.04) • 10^6	0.333 (±0.007)	158	0.012 (±0.001)	0.025 (±0.001)	106	48
mAb 37.1-wt	CD16F	2.10 (±0.04) • 10^5	0.253 (±0.005)	1,205	0.110 (±0.001)	0.004 (±0.001)	39	28
mAb 37.1		2.06 (±0.01) • 10^6	0.057 (±0.001)	28	0.015 (±0.001)	0.019 (±0.001)	16	51
Rituximab		2.88 (±0.05) • 10^5	0.624 (±0.008)	2,167	0.105 (±0.001)	0.004 (±0.001)	77	67
Immobilized ligand	Soluble analyte	k_a [$M^{-1} s^{-1}$]	k_d [s^{-1}]	K_D [pM]				R_{max} [RU]
mAb 37.1-wt	CD64	1.22 (±0.01) • 10^6	2.00 (±0.01) • 10^{-4}	164				47
mAb 37.1		2.80 (±0.01) • 10^6	5.65 (±0.01) • 10^{-5}	20				55
Rituximab		1.49 (±0.01) • 10^6	2.17 (±0.01) • 10^{-4}	146				59

CD64 datasets were fitted to a “1:1 binding” model, CD16 datasets were fitted to a “two-state” model. $K_{Dtot} = (k_{d1}/k_{a1}) \bullet (k_{d2}/(k_{d2}+k_{a2}))$. Numbers in parentheses are standard errors calculated by Biacore T200 Evaluation Software 1.0. RU, resonance units.

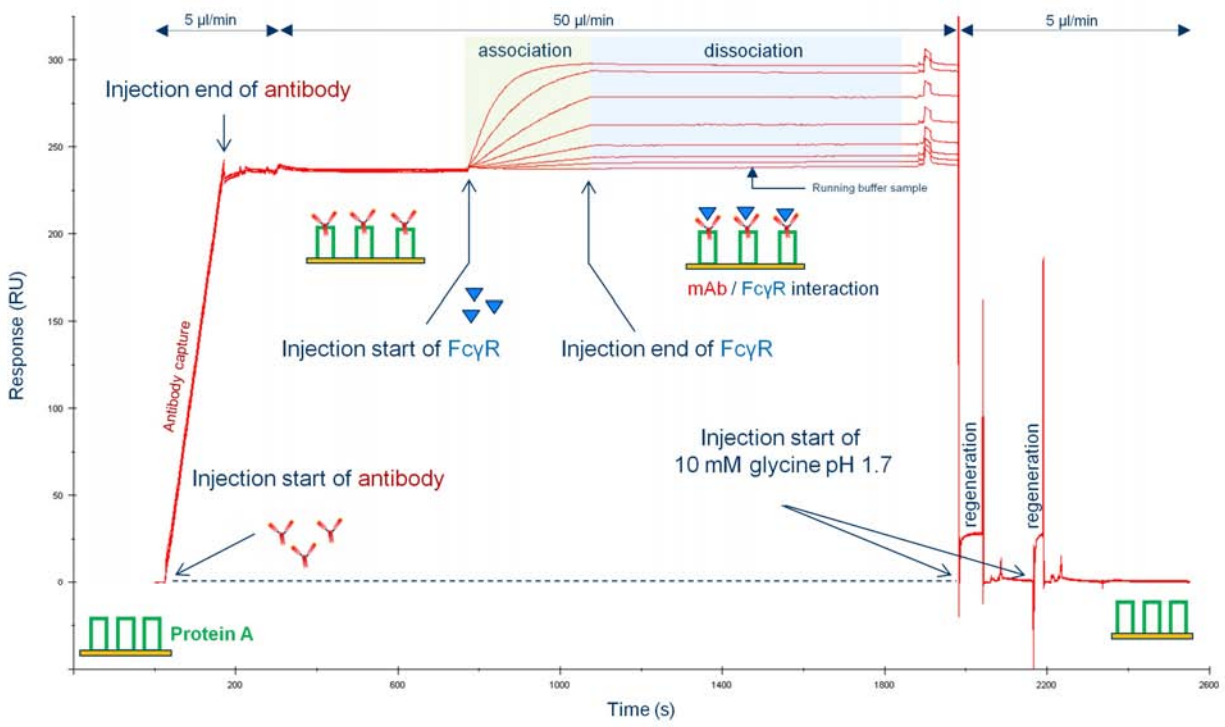
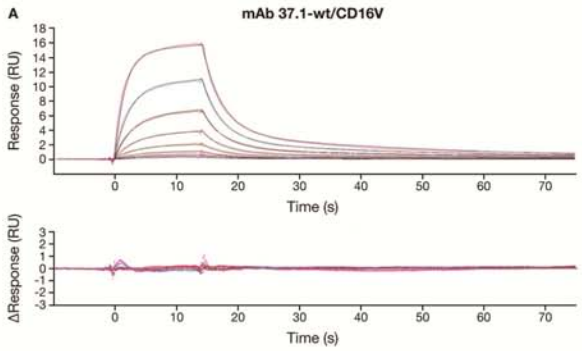


Figure S1. Kinetic SPR assay format

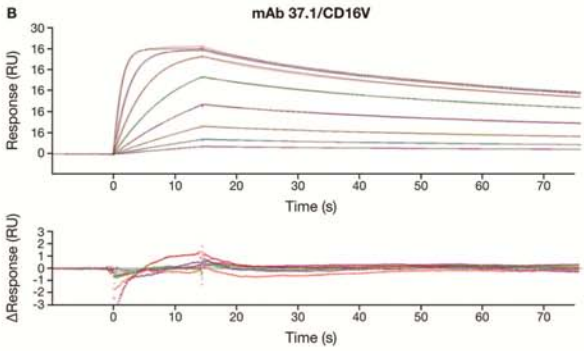
Overview of individual steps of the SPR assay format used in this study to kinetically characterize mAb/Fcγ receptor interactions. The overview was generated by combining schematic drawings with single referenced sensorgrams of an original titration series of soluble FcγR binding to Protein A captured mAb. RU, resonance units.

A



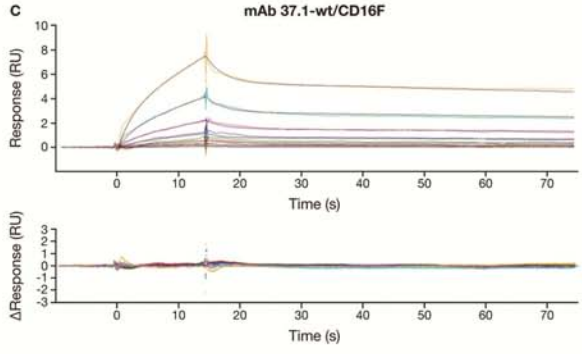
	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb 37.1-wt / CD16F	Fit model	Two-state; global; RI = 0
Analyte start conc. / Dilution factor	256 nM / 2x	R_{max}	27 RU
Number of injections for each analyte concentration	1	K_{D1}	215 nM
Flow rate	50 µl • min^{-1}	k_{a1}	1.57(2) • 10^{6} M^{-1} s^{-1}
Data collection rate	10 Hz	k_{d1}	0.338(4) s^{-1}
Running buffer	HBS-ET	χ^2	0.009 RU^2

B



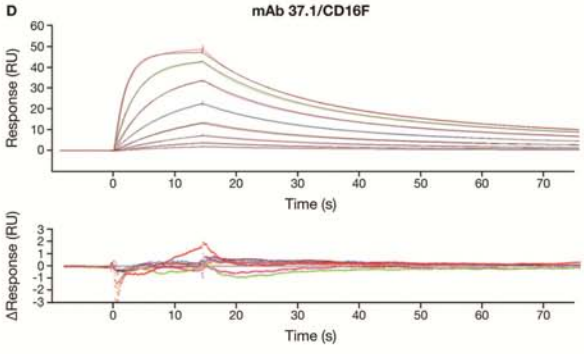
	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb 37.1/ CD16V	Fit model	Two-state; global; RI = 0
Analyte start conc. / Dilution factor	256 nM / 2x	R_{max}	51 RU
Number of injections for each analyte concentration	1	K_{D1}	4 nM
Flow rate	50 µl • min^{-1}	k_{a1}	4.12(1) • 10^{6} M^{-1} s^{-1}
Data collection rate	10 Hz	k_{d1}	0.016(1) s^{-1}
Running buffer	HBS-ET	χ^2	0.113 RU^2

C



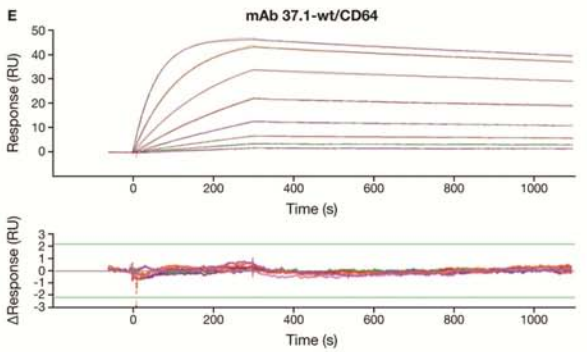
	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb 37.1-wt/ CD16F	Fit model	Two-state; global; RI = 0
Analyte start conc. / Dilution factor	256 nM / 2x	R_{max}	28 RU
Number of injections for each analyte concentration	1	K_{D1}	1205 nM
Flow rate	50 µl • min^{-1}	k_{a1}	2.10(4) • 10^{5} M^{-1} s^{-1}
Data collection rate	10 Hz	k_{d1}	0.253(5) s^{-1}
Running buffer	HBS-ET	χ^2	0.015 RU^2

D



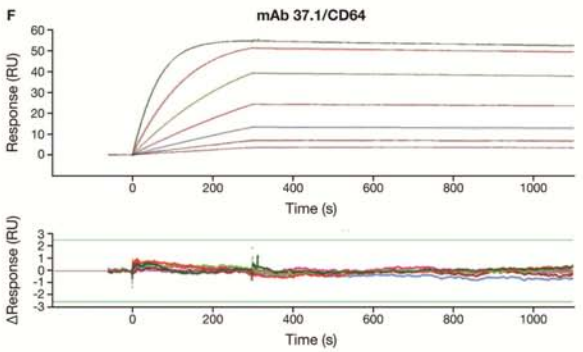
	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb 37.1/ CD16F	Fit model	Two-state; global; RI = 0
Analyte start conc. / Dilution factor	256 nM / 2x	R_{max}	51 RU
Number of injections for each analyte concentration	1	K_{D1}	28 nM
Flow rate	50 µl • min^{-1}	k_{a1}	2.06(1) • 10^{6} M^{-1} s^{-1}
Data collection rate	10 Hz	k_{d1}	0.057(1) s^{-1}
Running buffer	HBS-ET	χ^2	0.187 RU^2

E



	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb 37.1-wt/ CD64	Fit model	1:1 binding, global; RI = 0
Analyte start conc. / Dilution factor	15.9 nM / 2x	R_{max}	47 RU
Number of injections for each analyte concentration	1	K_D	164 pM
Flow rate	50 µl • min^{-1}	k_a	1.22(1) • 10^{6} M^{-1} s^{-1}
Data collection rate	10 Hz	k_d	2.00(1) • 10^{-4} s^{-1}
Running buffer	HBS-ET	χ^2/ U value	0.043 RU^2/1

F



	Setup		Measured parameters
Chip/Ligand/Analyte	Protein A / mAb_37.1/ CD64	Fit model	1:1 binding, global; RI = 0
Analyte start conc. / Dilution factor	15.9 nM / 2x	R_{max}	55 RU
Number of injections for each analyte concentration	1	K_D	20 pM
Flow rate	50 µl • min^{-1}	k_a	2.80(1) • 10^{6} M^{-1} s^{-1}
Data collection rate	10 Hz	k_d	5.65(1) • 10^{-5} s^{-1}
Running buffer	HBS-ET	χ^2/ U-value	0.068 RU^2/3

Figure S2. Kinetic SPR analyses of mAb/Fcγ receptor interactions

Kinetic analyses of soluble Fcγ receptors CD16V (A, B), CD16F (C, D) and CD64 (E, F) interacting with Protein A captured mAb 37.1-wt (left-hand-side panels) and mAb 37.1 (right-hand-side panels). Colored, double-referenced sensorgrams of mAb/CD16 interactions (A-D) are overlaid with black colored fits of a ‘‘two-state’’ model, while sensorgrams of mAb/CD64 interactions (E and F) are overlaid with fits of a “1:1 binding” model. Residual plots presented below each sensorgram series highlight the deviation (Δ) of data points from curve fit values and the actual fitting range. The table below each data set contains additional information about setup details (black text), the applied fitting model (red text) and measured parameter values (blue text).

RU, resonance units.

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Apoptosis

For determination of apoptosis using Annexin V staining, 100 µL of cells, at a density of 1×10^6 cells/mL in culture medium (RPMI 1640 with 10% FCS), were seeded into a 96-well round-bottom plate. 100 µL of antibody dilutions and controls (in culture medium) were added to the cells. Incubation was performed at 37°C in a humidified CO_2 incubator for 20–24 hours. Thereafter, 100 µL supernatant was removed from each well. Staining for apoptotic cells was performed using the Vybrant™ Apoptosis Assay Kit 2 (Invitrogen # V13241). 5 µL Alexa Fluor® 488 Annexin V (Component A) and 1 µL propidium iodide (PI) (100 µg/mL PI stock 1:10 diluted with Annexin V binding buffer) were added to the cells and incubated for 15 minutes at room temperature in the dark. The stained cells were transferred into FACS tubes (Falcon #352052) and 400 µL ice-cold Annexin V binding buffer was added to each tube. Samples were placed on ice and immediately subjected to FACS analysis using a BD FACS Canto™ Flow Cytometer.

ADCC

Effector cells were co-cultivated with target cells in presence of antibody in 96-well round-bottom microtiter plates in a final volume of 200 µL assay medium per well consisting of 10% human AB serum and 1% BSA in RPMI in 1:1 ratio. In each experimental run two plates were assayed in parallel, with duplicate data points on each plate. First effector cells were plated, followed by target cells and antibody solution. As a control, effector cells are cultivated in assay medium alone (effector cell control) and target cells are cultivated either in assay medium alone (spontaneous lysis) or in assay medium supplemented with 1% Triton X-100 (maximal lysis). The co-culture was incubated at 37°C in a humid CO_2 incubator for 3 hours. Cytotoxicity Detection Kit (LDH Roche #11 644 793 001) was used to determine cytotoxicity caused by antibody incubation.

Mouse strains

All mice were bred by and purchased from Taconic, Demark. The mice used for pharmacokinetic and pharmacodynamic studies were 7-week-old transgenic B6-CD37tm2065.Arte – TG(ACTB-Flpe)2Arte mice, housed in isolated-ventilated cages and were handled under sterile conditions in a laminar airflow cabinet. The mice used for *in vivo* antitumor efficacy studies were 8–10 week-old athymic female c.Cg/AnTac-Foxn1nuN20 strains. After arrival in the animal facility, mice were allowed to adjust to the new environment for at least 3 days before they were used for experiments. The animals were housed under standardized conditions (temperature 21.5 ± 1.5°C and 55 ± 10% humidity) in groups of 5 in Macrolon® type II cages.