Supplemental Materials and Methods

Materials

Rituximab and GA101 (obinutuzumab) Fc variants were provided by Hoffmann-La Roche AG (Basel, Switzerland). Variants of rituximab and obinutuzumab were generated by transient expression and purified via Protein A affinity chromatography followed by size exclusion chromatography. Quality control, confirmation of identity and absence of aggregates were performed by capillary electrophoresis/SDS-PAGE, mass spectrometry and analytical size exclusion chromatography.

Methods

IgG-FcγR interaction after <Fab> capturing

The SPR interaction analysis of captured IgG1 Fc variants and monomeric or dimeric FcγRs was performed on a Biacore T200 system (GE Healthcare) using medium densities of immobilized F(ab')2 Fragment Goat Anti-Human IgG, F(ab')2 fragment specific (Jackson ImmunoResearch code 109-006-006). Immobilization of the anti-Fab was performed on a CM5 chip using the standard amine coupling kit (GE Healthcare) at pH 4.5. The immobilization level of anti-Fab reached 400 RU. IgG1 Fc variants were prepared in solutions of 50 nM using running buffer 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20 (HBS-P+) and were captured with a pulse of 90 s at a flow rate of 10 µl/min. Subsequently, monomeric or dimeric FcγRs (FcγRs = FcγRIa, FcγRIIb, FcγRIIa (R131) and FcγRIIa (H131), FcγRIIIa (V158)) were applied at a concentration of 100 nM in HBS-P+ and a flow rate of 20 µl/min for 90 s. The dissociation phase was monitored for 300 s. The surface was regenerated two times by a 30-s washing step with a 10-mM NaOH at a flow rate

of 30 µl/ min. The stabilization period was set to 60 s. The Biacore T200 evaluation software was used for data analysis. The dimeric FcγRs have been engineered by a C-terminal Fc fusion to the extracellular domain of FcγRIIa and FcγRIIIa, utilizing again the P329G mutation in combination with L234A/L235A to prevent interaction with Fc receptors used in the assays.

Cell culture

Z138 (gift from University of Leicester) cells were cultivated in RPMI1640 containing 10% FCS and 1% Glutamax (Invitrogen/Gibco # 35050-038). SU-DHL-4 cells (DSMZ) were cultivated in DMEM (Invitrogen/Gibco #42430-082) + 10% FCS + 1% Glutamax.

Assessment of Cell Death

The induction of cell death by GA101 and rituximab Fc variants was tested using CD20-expressing mantle cell lymphoma (Z-138). Briefly, cells were harvested, counted, checked for viability and re-suspended at 0.526 x 10^6 cells/ml in RPMI1640 + 10 % FCS + 1 % Glutamax. 190 µl of cell suspension (containing 0.1 x 10^6 cells) were incubated in round-bottom 96-well plate for 20 h - 24 h at 37°C 5 % CO_2 in the cell incubator with different concentrations of the GA101 or rituximab variants (16 ng/ml - 10 µg/ml). Afterwards, the cells were washed once with Annexin V Binding Buffer (10 mM HEPES/NAOH pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) before incubation for 30 min at 4°C in the dark with 100 µl/well Annexin V FLUOS (Roche #11828681001, pre-diluted in Annexin V Binding Buffer 1:75). The cells were washed by addition of 80 µl/well Annexin V Binding Buffer and immediately

analyzed by FACS using a FACS CantoII (Software FACS Diva) after addition of pre-diluted PI solution (Sigma Aldrich #P4864, 1:4000).

Assessment of CDC

SU-DHL-4 and Z-138 cells were plated in AIM V® medium at a density of 5×10^4 cells/well in flat-bottomed 96-well plates. Diluted anti-CD20 antibody (end concentrations 100-0.01 µg/ml) was added to cells 10 minutes before the addition of Low-Tox® Rabbit Complement preparation (Cedarlane Laboratories Ltd). CDC was estimated by measuring LDH release in cell supernatants 2 hours after incubation at 37°C in 5% CO_2 as well as based on Alamar Blue (Biosource) staining after 18-24 h incubation.

CDC was calculated using the following formula:

$$\text{Percentage CDC} = \left(\left[\frac{\text{sample release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \right] \right) \times 100.$$

Spontaneous lysis, corresponding to LDH released by target cells or AlamarBlue OD in the absence of antibody, was defined as 0% CDC, with maximal lysis (target cells lysed with 1.3% Triton X-100) defined as 100% CDC. The average percentage of CDC and standard deviations of the triplicates of each experiment were calculated.

ADCC

Peripheral blood mononuclear cells (PBMCs) were isolated from Buffy Coats obtained from the Blutspendezentrale Zürich or healthy volunteering blood donors from Hoffmann-La Roche AG. Briefly, blood was diluted with phosphate buffered saline (PBS) and separated by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich).

Z-138 and SU-DHL-4 target cells were harvested, washed, resuspended in AIM V® medium (Life Technologies), and plated at a concentration of 0.25×10^4 cells/well into 96-U-bottom

plates. The respective anti-CD20 antibody dilutions were added to cells (final assay end concentrations 0.01-1000 ng/ml) as well as pre-diluted anti-CD107 a FCAS ab (PE, Biolegend) and incubated for 10 minutes before addition of the effector cells (PBMCs, 0.625 x 10^6/well). Effector (E) and target (T) cells were then incubated for 4 hours at 37°C (triplicates for all samples). Lactate dehydrogenase (LDH) release was measured using the LDH Cytotoxicity Detection Kit (Roche Applied Science). ADCC was calculated using the following formula:

$$\text{Percentage ADCC} = \left(\left[\frac{\text{sample release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \right] \right) \times 100.$$

Spontaneous release, corresponding to target cells incubated with effector cells without antibody, was defined as 0% cytotoxicity, with maximal release (target cells lysed with 1% Triton X-100) defined as 100% cytotoxicity. The average percentage of ADCC and standard deviations of the triplicates of each experiment were calculated.

After removing supernatant for LDH determination, the remaining cells were washed with FACS Buffer and stained with anti-CD3 (PE/Cy7, BD Biesciences), anti-CD56 (APC, Biolegend) and anti-CD16 (FITC, Dako) for 30 min at 4°C in the dark. Unbound FACS ab was removed by washing with FACS Buffer before treating the samples with FACS Lysing solution (BD Biosciences). Afterwards, the expression of CD16 and CD107a on CD3-CD56+ NK cells were determined by flow cytometry using a FACSCantoII (BD Biosciences).

B cell depletion and cytokine release in Human Healthy Whole Blood

Fresh blood from healthy volunteers was collected in heparin-containing syringes. Blood aliquots (190 µl/well) were placed in deep-well 96-well plates, supplemented with anti-CD20

antibody dilutions (20 µL/well, final assay end concentrations 0.3-1000 ng/ml) and incubated for 20-24 hours at 37°C in 5% CO_2 in a humidified cell incubator. After incubation, 35 µl/well blood were removed and incubated with 20 µl/well pre-mixed anti-CD45-APC (BD Biosciences), anti-CD3-PE/Cy7 and anti-human CD19-PE (both Biolegend) for 15 minutes at room temperature (in the dark) to estimate the total, T- and B-lymphocyte populations, respectively. Subsequently, 200 µl/well of FACS lysis solution (BD Biosciences) was added to deplete erythrocytes and to fix cells prior to flow cytometry. Results were evaluated by gatingCD45-positive cells and determining the CD3-positive T-cell and CD19-positive B-cell populations therein.

B-cell depletion was evaluated using antibody-untreated samples as a 100% control and the following formula:

$$100 - \left(\left[\frac{100}{\text{B/T cell ratio in control}} \right] \right) \times \left(\left[\text{B/T cell ratio in sample containing antibody} \right] \right).$$

The average B-cell depletion and standard deviations of the triplicates of each experiment were calculated.

Cytokine release was determined by collecting part of the serum after 20-24 h and performing a CBA analysis determining the concentrations of IL-6, IL-8, TNF and IFNγ by following the instructions of the manufacturer (BD Biosciences).

In vivo antitumor activity

The human DLBCL cell line SU-DHL4 was subcutaneously inoculated (5×10^6 cells) with Matrigel™ (BD Biosciences) into the right flank of 6–7-week-old female SCID beige mice (Charles River). Animals were maintained under specific pathogen-free conditions according

to international guidelines. Continuous health monitoring was carried out on a regular basis, with daily monitoring of general condition and behaviour. Primary tumor volume (TV) was calculated according to the National Cancer institute (NCI) protocol

$[\mathrm{TV} = (\mathrm{length} \times \mathrm{width}^2)/2]$, where "length" and "width" are the long and short diameters of the tumor mass in millimeters. Antitumor activity was assessed by calculating tumor growth inhibition (TGI) based on medians using the following formula:

$$100 - \frac{median(TV(treated)_{dayz} - TV(treated)_{dayx})}{median(TV(resp.control)_{dayz} - TV(resp.control)_{dayx})} \times 100$$

At 21 days after cell transplantation, 10 animals each with established subcutaneous (s.c.) SU-DHL4 tumors (approx. 300 mm^3) were randomized to vehicle control, GA101, GA101-WT, GA101-P329GLALA, rituximab, or rituximab-P329GLALA (all antibodies were administered at 30 mg/kg i.p.). Treatment commenced 21 days after tumor-cell inoculation (median tumor volume, 296–302 mm^3), with administration repeated on days 28, 35, and 42.

Tumor size was determined at the indicated time points and tumor growth inhibition (TGI) and non parametric treatment-control ratios (np TCR) with 95% confidence interval as well as the number of tumor free animals at study termination on Day 49 after tumor-cell inoculation were determined.[1] Raw data from the experiment were processed in the statistics software SAS-JMP version 8.0.2.2 (SAS, 2007) using the menu *RocheTools 3.1*. Primary tumor volume and antitumor activity were calculated using established methods.

Supplemental Reference

1. Fieller E: **Some problems in interval estimation.** *J Royal Stat Soc* (1954) **B16**

Supplemental Figure Legend

Supplemental Figure 1: Binding to FcgRI, FcgRIIa and FcgRIIIa as determined by surface plasmon resonance. A: FcgRI interaction; B: FcgRIIa dimer interaction; C: FcgRIIIa dimer interaction The SPR interaction analysis of anti F(ab')2 captured IgG1 Fc variants and monomeric or dimeric Fc gamma receptors was performed on a Biacore T200 system (GE Healthcare) using high densities of immobilized AffiniPure F(ab')2. IgG1 Fc variants were prepared in solutions of 50 nM. Subsequently FcγRIa mono, FcγRIIa dimer and FcγRIIIa dimer were applied at a concentration of 100 nM in PBS-P+. For data analysis the sensograms were compared to each other.

Supplemental Figure 1