GA101 P329GLALA, a variant of obinutuzumab with abolished ADCC, ADCP and CDC function but retained cell death induction, is as efficient as rituximab in B-cell depletion and antitumor activity

CD20-antibodies are believed to mediate three different mechanisms of action (MOA): 1) direct cell death (DCD)/apoptosis, 2) antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP), 3) complement-dependent cytotoxicity (CDC). The relative contribution of these MOAs to the clinical efficacy remains unconfirmed. Here, we investigated the contribution of these MOAs to the preclinical efficacy of obinutuzumab and rituximab using Fc-immune effector function inactive variants by introduction of P329GLALA mutations. We show in whole blood B-cell depletion and a xenograft model that DCD and ADCC/ADCP are required for the anti-tumoral efficacy of obinutuzumab. Most notably, the P329G LALA variant of obinutuzumab, that completely lacks Fc-mediated effector functions, but has retained DCD induction, is as efficacious as rituximab in B-cell depletion and *in vivo* anti-tumor activity supporting the role of DCD for the efficacy of obinutuzumab.

Obinutuzumab (GA101) is a glycoengineered Type II antibody[1] and, to date, is the only glycoengineered antibody approved in the US and Europe for first-line treatment of chronic lymphocytic leukemia (CLL),[2,3] for treatment of patients with follicular lymphoma refractory to/relapsed of a rituximab containing regimen combined with bendamustine,[4] and for first-line treatment of follicular lymphoma in Europe.[5] Obinutuzumab is believed to work primarily through DCD induction and ADCC/ADCP; both MOAs were designed to be enhanced compared to rituximab, whereas it exhibits reduced CDC by virtue of being a Type II CD20-antibody.[6,7] However, the relative contribution of these different MOAs to the overall anti-tumoral efficacy is unproven. In order to further dissect the MOA, immune effector inactive variants of obinutuzumab and rituximab were assessed *in vitro* and *in vivo*.

For this purpose, glycoengineered GA101 (GA101 GE), non-glycoengineered wildtype GA101 (GA101-WT), rituximab, and effector-dead versions of GA101 (GA101-P329GLALA) and rituximab (rituximab-P329GLALA) with abolished FcγR and C1q binding were generated by introduction of P329G/Leu234Ala/Leu235Ala (P329GLALA) Fc-mutations.[8]

All variants were quality controlled by SDS-page, size exclusion chromatography, mass spectrometry and bind-

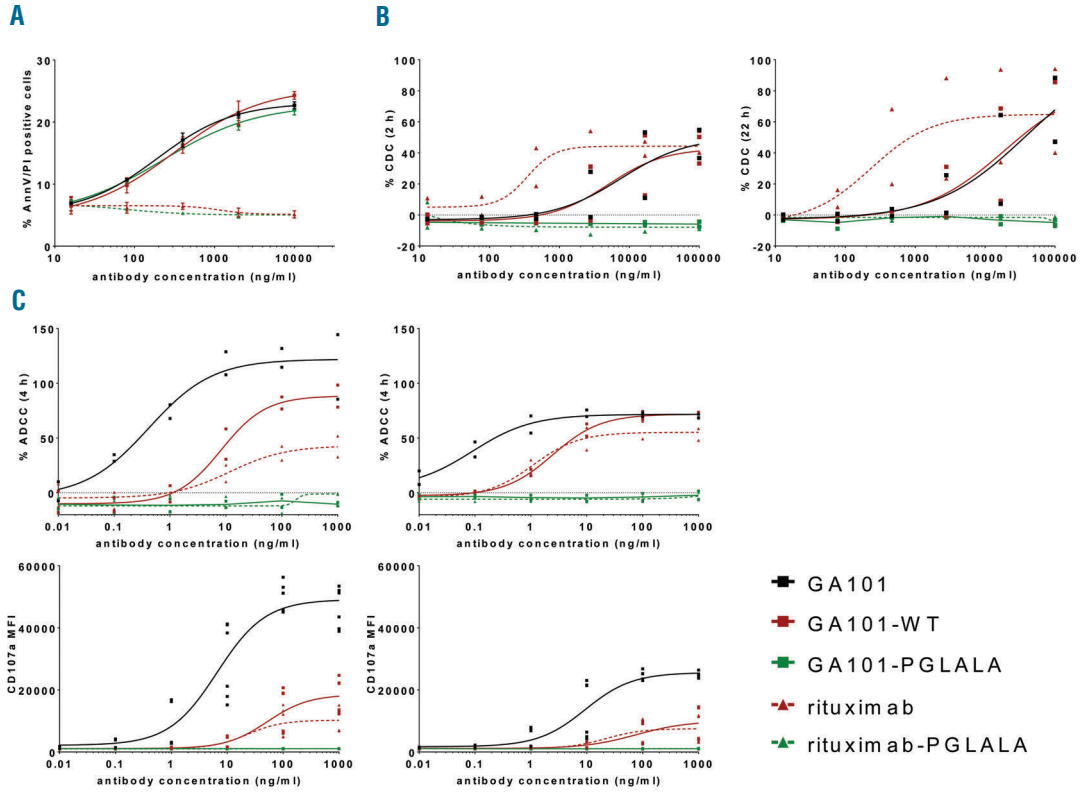

Figure 1. *In vitro* characterization of GA101, rituximab and effector inactive variants. A: P329GLALA mutations do not affect direct cell death. Z-138 cells were incubated for 22 h in the presence of CD20 antibodies as indicated. Afterwards, cells were stained with Annexin V FLUOS and PI and analysed by flow cytometry. B: P329GLALA mutations abolish CDC induction. SU-DHL-4 or Z-138 cells were incubated with rabbit complement in the presence of CD20 antibodies as indicated. CDC was calculated based on LDH release after 2 h (left) or AlamarBlue readout after 22 h (right). Average triplicates of both cell lines shown. C: P329GLALA mutations abolish ADCC induction and NK cell activation, SU-DHL-4 (right) or Z-138 (left) cells were incubated with human PBMCs (E:T 25:1) for 4 h in the presence of CD20 antibodies as indicated. Top: ADCC was calculated based on LDH release, bottom: Degranulation of $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry measuring surface CD107a expression levels. Triplicates (LDH: Average triplicates) for 2 donors per target cell line shown.

ing to CD20+ cell lines (*data not shown*). Surface plasmon resonance analysis demonstrated that GA101-P329GLALA and rituximab-P329GLALA display only residual FcγRI binding activity in an assay utilizing captured antibody and the recombinant FcγRI receptor in solution (*Online Supplementary Figure S1A*). To mimic the avidity of the low affinity Fcy receptors FcγRII and FcγRIII, the same assay using dimeric FcγRII and FcγRIII was used to demonstrate Fc silencing in a more sensitive setting.[8] GA101-P329GLALA and rituximab-P329GLALA did not show any detectable FcγRIIa or FcγRIIIa binding (*Online Supplementary Figure S1B-C*), while FcRn binding and pharmacokinetics were not affected by these mutations.[8]

All antibody variants retained cell death inducing properties of the respective parental antibody as measured by AnnexinV/PI flow cytometry using the Z-138 lymphoma cell line, with GA101 and variants mediating superior overall cell death induction as compared to rituximab and variants (Figure 1A).[1] In CDC, ADCC and NK cell activation assays using Z-138 and SU-DHL4 lymphoma cell lines, P329GLALA variants of GA101 and rituximab did not mediate CDC (Figure 1B) or ADCC (Figure 1C, top) or NK cell activation (Figure 1C, bottom). As previously demonstrated, GA101 and GA101-WT as Type II CD20 antibodies mediated inferior CDC compared to the Type I CD20-antibody rituximab (Figure 1B).[9] Vice versa, GA101 showed superior potency in ADCC and NK cell activation compared to the non-glycoengineered wild-type antibodies (Figure 1C) due to higher FcγRIIIa affinity as a consequence of glycoengineering, and likely related to reduced CD20 internalization induced by Type II compared to Type I CD20 antibodies.[10] Taken together, the introduction of P329GLALA mutations into the Fc portion abolished Fc-mediated ADCC and CDC effector functions while DCD induction was not affected.

Subsequently, we investigated the activity of GA101, rituximab, and effector function inactive variants for depletion of CD19+ B cells in the whole blood from healthy volunteers by flow cytometry,[9,11] an assay integrating DCD, ADCC, ADCP and CDC. In whole blood assays, GA101 demonstrated maximal B-cell depletion in terms of potency and absolute B-cell depletion followed by GA101-WT and rituximab (Figure 2A). Most notably, GA101-P329GLALA retained significant B-cell depletion, while rituximab-P329GLALA did not mediate B-cell depletion. Thus, in whole blood B-cell depletion, ADCC/ADCP appears to play an important role as shown previously,[12] but apparently Type II-related enhanced DCD induction is important for the MOA of GA101 as well, whereas DCD does not appear to contribute significantly to B-cell depletion by rituximab.

One consequence of glycoengineering obinutuzumab may be the higher incidence of cytokine related infusion reactions, most likely due to enhanced activation of immune effector cells through FcγRIIIa and subsequent cytokine release upon binding to CD20 on peripheral and/or leukemic B cells.[13] Therefore, we investigated the release of IFN-γ, IL-6, IL-8 and TNF-α following administration of GA101, rituximab, and effector function inactive variants in whole blood using a bead-based CBA immunoassay. In spite of variability between healthy donors, GA101 had the strongest effect on cytokine release followed by GA101-WT. Rituximab showed reduced cytokine release compared to GA101, but also compared to GA101-WT, whereas no cytokine release above baseline was observed with GA101-P329GLALA (Figure 2B). These data indicate that cytokine release does not originate from direct Type II mediated mechanism such as DCD, but from immune effector cells as a consequence of enhanced Fc-FcγRIII interaction. Notably, cytokine release by GA101-WT is stronger than that of rituximab which implies that Type II mediated mechanism, e.g., reduced CD20 internalization,[10] may contribute to sustain ADCC/ADCP and stronger cytokine release.

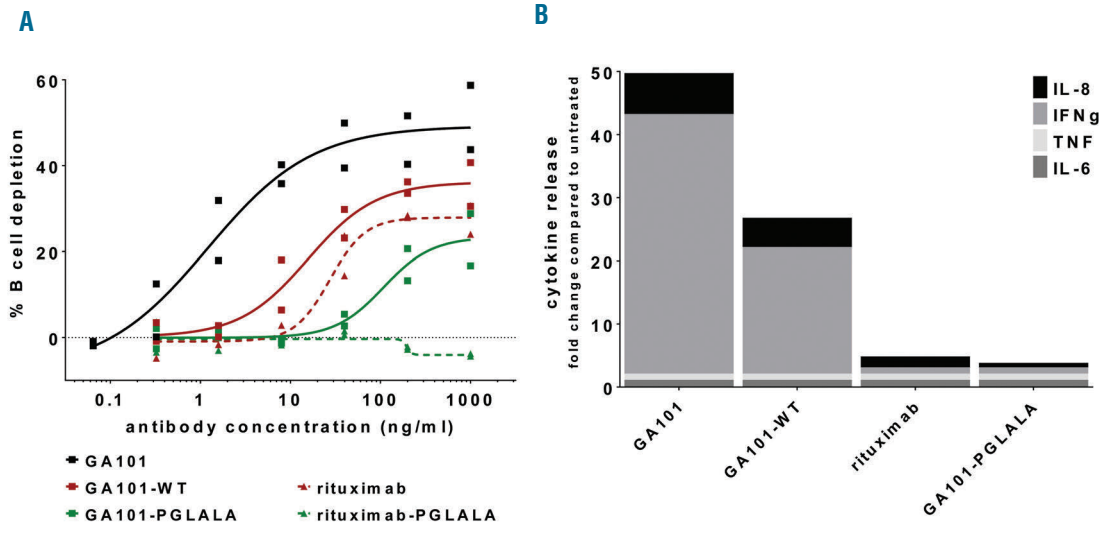

Figure 2A. Immune effector-inactive GA101-P329GLALA mediates significant whole blood B-cell depletion. B-cell depletion in whole blood of healthy donors was determined after 20h incubation in the presence of CD20 antibodies as indicated by flow cytometry. Summary results (average triplicates) from two different blood donors are shown. B: Absence of cytokine release for GA101-P329GLALA. Cytokine release upon incubation of human whole blood with CD20 antibodies was measured after 20h incubation performing a CBA analysis of the plasma. Mean fold change of cytokine release compared to untreated blood shown (N = 3).

Finally, we tested the *in vivo* efficacy of selected variants in a s.c. SU-DHL4 xenograft model in SCID beige, an *in vivo* model integrating DCD, monocyte/macrophage-mediated ADCC/ADCP and CDC. GA101-P329GLALA had typical IgG-like pharmacokinetics with a clearance of 0.13 mL/h/kg and a t1/2 of 23 days confirming that these mutations do not affect pharmacokinetics. GA101 (TGI > 100%, non-parametric TCR (npTCR) 0, 95% CI 0-0) and non-glycoengineered GA101-WT (TGI > 100%, npTCR 0, 95% CI 0-0.006) showed equivalent efficacy with complete tumor remission in 9/10 mice for GA101 and 8/10 mice for GA101-WT, while rituximab was only able to slow down tumor progression (TGI 71%, npTCR 0.37, 95% CI 0.29-0.50) and no tumor-free animals were observed. Most notably, GA101-P329GLALA, which is completely devoid of Fc-mediated effector functions and relies on DCD induction, mediated significant anti-tumoral efficacy (TGI 62%, npTCR 0.43, 95% CI 0.30-0.60) comparable to rituximab, while rituximab-P329GLALA had only residual anti-tumoral activity (TGI 26%, npTCR 0.73, 95% CI 0.44-0.92) (Figure 3). The comparable efficacy of GA101 and GA101-WT on the one hand shows that complete tumor remission in this model does not depend on the enhanced interaction of the glycoengineered Fc-part with muFcγRIV, the homologue of human FcγRIIIa, which is expressed on murine macrophages, monocytes and neutrophils, but not NK cells.[14] However, as GA101-WT is superior in efficacy to GA101-P329GLALA, ADCC/ADCP-related immune effector functions are important to achieve complete tumor remissions. The superiority of GA101-WT over rituximab, which both carry the same non-glycoengineered Fc-portion, on the other hand has to be attributed to Type II-related activities resulting in enhanced DCD and ADCC/ADCP due to reduced CD20 internalization.[10] As SCID beige mice have disabled NK cells, and murine NK cells do not express muFcγRIV, this model does not fully reflect the human immune system. Thus, in humans, an additional contribution of NK-cell mediated ADCC can be expected.

Overall, the data indicate that in whole blood B-cell depletion and the SU-DHL4 xenograft model, both DCD and ADCC/ADCP are required for maximal anti-tumoral efficacy of obinutuzumab. Most notably, the effector dead version of obinutuzumab which lacks ADCC, ADCP and CDC activity, but has retained DCD induction, is as efficacious as rituximab, which strongly supports the important role of direct Type II-related DCD for the MOA and efficacy of obinutuzumab in preclinical models. *Vice versa*, the data demonstrate the major importance of Fc-mediated immune effector functions (ADCC, ADCP and CDC) for the MOA of rituximab.[15]

While use of a P329GLALA variant of obinutuzumab in clinical trials to elucidate the clinical MOA of obinutuzumab would be most informative, but impossible from practical and ethical considerations, it is highly likely, based on these findings, that both MOAs, Type II mediated DCD and ADCC/ADCP, significantly contribute to the clinical MOA of obinutuzumab. Further biomarker studies may shed light onto the clinical MOA and whether e.g., obinutuzumab may provide benefit in patients with a compromised innate immune system. Furthermore, these data suggest the application of effector inactive variants of therapeutic antibodies in the effort to elucidate their MOA in preclinical models. Ultimately, the P329GLALA variant of obinutuzumab is expected to no longer mediate cytokine-related infusion reactions, but retain significant B-cell depletion. While such an antibody may not be applied for the treatment of B-cell malignancies, it may have therapeutic value e.g., in patients with autoimmune diseases.

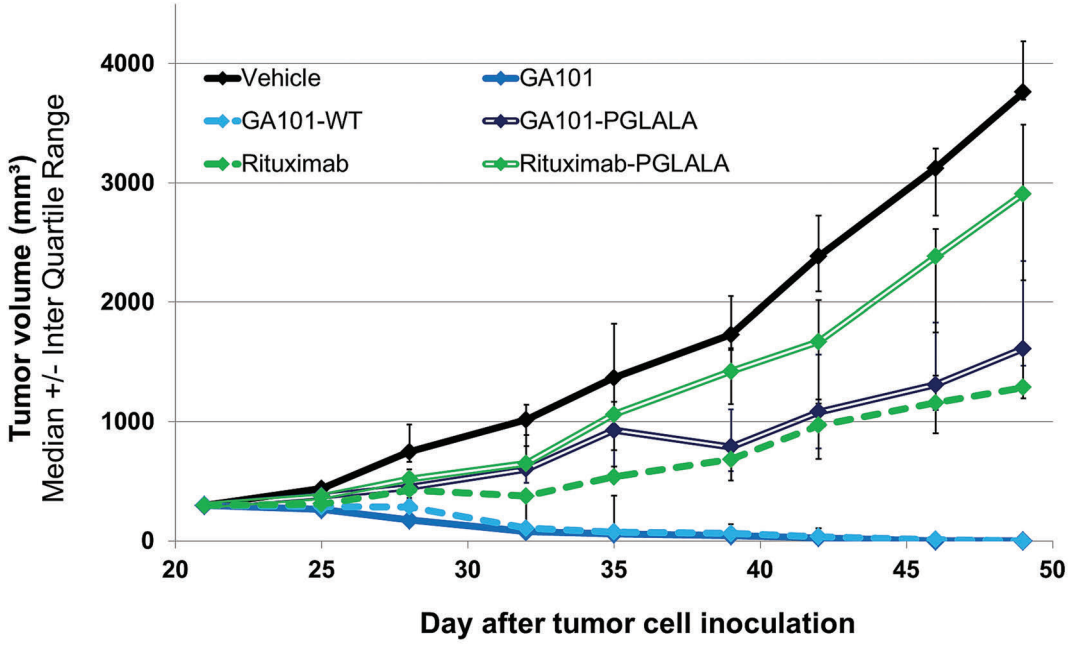

Figure 3. Immune effector-inactive GA101-P329GLALA mediates comparable anti-tumoral efficacy compared to rituximab in s.c. SU-DHL4 xenograft model in Scid beige mice. Scid beige mice bearing established 300 mm^3 s.c. SU-DHL4 tumors were treated with the indicated antibodies (30 mg/kg, q7dx4; IP). Tumor size was determined at the indicated time points, and tumor growth inhibition 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 were determined.

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