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Comparative Assessment of Clinically Utilized CD20-Directed Antibodies in Chronic Lymphocytic Leukemia Cells Reveals Divergent NK Cell, Monocyte, and Macrophage Properties

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CD20 is a widely validated, B cell–specific target for therapy in B cell malignancies. Rituximab is an anti-CD20 Ab that prolongs survival of chronic lymphocytic leukemia (CLL) patients when combined with chemotherapy. Ofatumumab and GA101 (obinutuzumab) are CD20-directed Abs currently being developed as alternative agents to rituximab in CLL based upon different properties of enhanced direct cell death, NK cell-mediated Ab-dependent cellular cytotoxicity, or complement-dependent cytotoxicity. Despite widespread study, ofatumumab and GA101 have not been compared with each other, nor studied for their interactions with monocytes and macrophages which are critical for the efficacy of anti-CD20 Abs in murine models. In CLL cells, we show that direct cell death and complement-dependent cytotoxicity are greatest with GA101 and ofatumumab, respectively. GA101 promotes enhanced NK cell activation and Ab-dependent cellular cytotoxicity at high Ab concentrations. Ofatumumab elicits superior Ab-dependent cellular phagocytosis with monocyte-derived macrophages. GA101 demonstrated reduced activation of monocytes with diminished pERK, TNF-α release, and FcγRIIa recruitment to lipid rafts. These data demonstrate that GA101 and ofatumumab are both superior to rituximab against CLL cells via different mechanisms of potential tumor elimination. These findings bear relevance to potential combination strategies with each of these anti-CD20 Abs in the treatment of CLL. The Journal of Immunology, 2013, 190: 000–000.

Expression of CD20 glycoprotein is tightly restricted to the surface of B cells, making it an ideal therapeutic target for Ab therapy. Over the past decade, CD20 has become a well-validated target for therapy in B cell malignancies, mainly because of the approval of rituximab for non-Hodgkin lymphoma (NHL) in 1997. Rituximab is a chimeric mAb that has revolutionized therapy in a variety of B cell malignancies, including chronic lymphocytic leukemia (CLL). In CLL, rituximab was shown to have modest single-agent activity (reviewed in 1, 2), but has shown greatest promise in combination with chemotherapy (chemoimmunotherapy), in which retrospective phase II comparison studies (3, 4) and a recent prospective phase III study demonstrated prolongation of survival (5). Despite its successes, not all patients respond to rituximab therapy and virtually all relapse. Improving the properties of rituximab to enhance its efficacy further is therefore highly desirable.

B cell depletion by rituximab and other anti-CD20 Abs has been proposed to occur via several mechanisms. Although many effector cells including NK cells, monocytes, macrophages, and granulocytes can mediate Ab-dependent cellular cytotoxicity (ADCC), several sentinel papers have revealed that B cell depletion with anti-CD20 or anti-CD19 Abs is predominantly dependent on monocytes and their expression of FcγRIIa, FcγRIIIa, and FcγRIV (6–8). Furthermore, others have shown that TNF-α secreted by monocytes activates NK cells, and this crosstalk mediates enhanced ADCC (9, 10). In humans, NK cells have been suggested to be most important for rituximab-mediated tumor clearance based on the FcγRIIa single-nucleotide polymorphisms expressed predominately in this cell type and result in a low- or high-affinity receptor that is highly predictive of Ab response (11, 12) and of normal B cell depletion (13). In CLL, these same FcγRIIa single-nucleotide polymorphisms have no correlation with response (14, 15) or extended progression-free survival (16). The true importance of NK cells, monocytes, or other effector cells to CD20 Ab-mediated killing in CLL remains controversial.

Other mechanisms of anti-CD20–mediated cytotoxicity, including direct cell death and complement-dependent cytotoxicity,
have also been documented. Direct cytotoxicity with type I anti-CD20 Abs such as rituximab generally requires cross-linking with an anti-Fc–directed Ab in vitro (17, 18), mimicking in vivo binding to FcγR on effector cells. Evidence of in vivo apoptosis following rituximab treatment in CLL cells has supported such crosslinking as a mechanism of action (19). However, a recent study has challenged this by using a novel mouse model with an FcγR lacking the active immune tyrosine-activating motif (ITAM) that demonstrated little in vivo activity with CD20 Abs (20). Type II anti-CD20 Abs lack the need for cross-linking and offer a potential advantage clinically by promoting homotypic adhesion and actin-dependent, lysosome-mediated cell death (21). Complement-dependent cytotoxicity (CDC) with rituximab occurs, but the Ag density on CLL cells limits killing by this mechanism (22, 23). In addition, upregulation of complement protection Ags CD55 and CD59 can occur after rituximab-based therapy (24, 25).

Based on the success of rituximab in NHL and CLL, the next generation of anti-CD20 therapeutic Abs is emerging, intelligently engineered to enhance the efficacy of anti-CD20 therapy via different mechanisms of action. Ofatumumab (Arzerra) is a human, type I Ab that uniquely binds to the small and large extracellular loop of CD20 (26). It has been shown to induce potent CDC in vitro compared with rituximab at low concentrations and low Ag density (26, 27). Clinically, ofatumumab produced responses in >50% of fludarabine- and alemtuzumab-refractory CLL patients with modest toxicity (28, 29) and is active in patients regardless of prior treatment with rituximab (30); it is currently approved for this indication. GA101 (obinutuzumab) is a type II humanized anti-CD20 Ab that promotes direct killing without in vitro cross-linking and has an afucosylated Fc domain engineered for enhanced FcγRIIIa binding (31–36). Direct cell death and ADCC by NK cells is superior with GA101 compared with rituximab against malignant B cells (31). A phase I study with GA101 in CLL showed favorable efficacy in relapsed and refractory patients with modest toxicity (37). The follow-up phase II study of 20 relapsed and refractory CLL patients demonstrated promising blood clearance of tumor cells, but only a 20% response by International Workshop on CLL 2008 response criteria (38). These included a 50% reduction of nodal mass, measured by computed tomography, as a criterion for partial response, which was not attained in the majority of patients. Prior studies with other therapeutic Abs have not mandated computed tomography for assessment of response, but have relied on physical examination measurements alone. Response in nodal disease may also have been influenced by dose of GA101, and larger studies will be required to ascertain the true efficacy of this Ab.

Whereas ofatumumab and GA101 have each been compared with rituximab in CLL, no study has compared these two Abs or examined each one’s interaction with monocytes or macrophages. In this study, we report a detailed analysis demonstrating that ofatumumab and GA101 are both superior to rituximab, but each has varied ability to activate different effector cell types that might bear relevance to combination-based strategies in CLL.

Materials and Methods

Patient sample processing and cell culture

Blood was obtained from patients who had given informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the institutional review board at The Ohio State University. All patients examined in this series had immunophenotypically defined CLL and had been without prior therapy for a minimum of 30 d at the time of collection. CLL PBMCs were isolated from freshly donated blood with Ficoll density gradient centrifugation (Ficoll-Plaque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL fractions were prepared with the use of the Rosette-Sep kit from Stemcell Technologies (Vancouver, BC, Canada) according to the manufacturer’s instructions. Isolated cells were incubated in RPMI 1640 (Life Technologies, Grand Island, NY) media supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), and 56 U/ml penicillin with 56 μg/ml streptomycin (Life Technologies) at 37°C in an atmosphere of 5% CO₂. Normal cells were obtained from Red Cross partial leukocyte preparations, and NK cells were negatively selected with Rosette-Sep kits (Stemcell Technologies) according to the manufacturer’s instructions. Monocytes were positively selected using the MACS system (Miltenyi, Cambridge, MA). The purity of enriched populations of normal cells was routinely checked with the use of CD19, CD56, and CD14-PE staining by flow cytometry. NK cell and monocyte purity usually ranged from 75–90%, and purity of B cells was usually >85% CD19⁺. Normal samples were from anonymous donors as part of a second exemption protocol approved by the institutional review board at The Ohio State University. The Raji and THP-1 cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% FBS. The NK92 CD16⁺ cells have been described previously (39).

In vitro treatment of cells with Abs

Cells were suspended in complete media at a density of 1 × 10⁶ cells/ml immediately after isolation. All therapeutic Abs were used at 10 μg/ml unless noted otherwise. Where indicated, the Fc-specific goat anti-human FcγR cross-linker (Jackson ImmunoResearch, West Grove, PA) was added to the cell suspension 5 min after adding the primary Abs, at a concentration 5-fold that of the primary Abs (i.e., 50 μg/ml for 10 μg/ml). In addition, a group of samples with no treatment was collected as media control.

Assessment of apoptosis by flow cytometry

The apoptosis of cells was measured using annexin V–FITC/PI staining followed by FACS analysis according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA) as described previously (40). Results are presented as percentage cytotoxicity, which is defined as (% annexin V⁻ and/or PI⁻ cells of treatment group) / (% annexin V⁻ and/or PI⁻ cells of media control) × 100. FACS analysis was performed using a Beckman Coulter FC500 cytometer (Beckman Coulter, Indianapolis, IN). Ten thousand events were collected for each sample, and data were acquired in list mode.

Complement dependent cytotoxicity

CLL B cells were suspended at 10⁶ cells/ml in RPMI 1640 media, media with 30% plasma from the patient blood samples, or media with 30% heat-inactivated (56°C, 30 min) plasma. Cells were then treated with Abs and incubated at 37°C for 1 h, pelleted, and resuspended in 1% formaldehyde with Live/Dead Stain (Sigma-Aldrich). The extent of CDC was measured using FACS analysis of percent staining for dead.

Ag quantification

Quantitative analysis of CD20 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, IN), according to the manufacturer’s instructions.

In vitro stimulation and cytokine assays

For in vitro NK cell stimulation experiments, wells of a 96-well flat-bottom plate were coated with 20 μg/ml of Ab in PBS. Freshly isolated NK cells were plated at 2 × 10⁶ cells/well in 96-well flat-bottom plate (BD Pharmingen) and were exposed to a range of concentrations of anti-CD20 Abs for 18 h in RPMI media supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD) and 1% l-glutamine (Life Technologies, Carlsbad, CA). Results are presented as percentage cytotoxicity, which is defined as (% annexin V⁻ and/or PI⁻ cells of media control) × 100. FACS analysis was performed using a Beckman Coulter FC500 cytometer (Beckman Coulter, Indianopolis, IN). Ten thousand events were collected for each sample, and data were acquired in list mode.

Real-time reverse-transcription PCR

Monocytes were stimulated for 18 h with immobilized Abs. Cells were collected, and total RNA was extracted using TRIzol (Life Technologies). Real-time PCR for TNF-α was performed using predesigned TaqMan Gene Expression Assays and ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

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ADCC assay

ADCC activity was determined by standard 4-h 51Cr-release assay. 51Cr-labeled target cells (5 × 10^3 cells/well of B CLL or Raji cells) were incubated for 30 min with various concentrations of Abs. Unbound Abs were washed off and cells were placed in 96-well plates. Effector cells (NK cells from healthy donors or CLL patients) were then added to the plates at the indicated E:T ratios. After 4 h of incubation, supernatants were removed and counted on a Perkin Elmer (Waltham, MA) Wizard γ counter. The percentage of specific cell lysis was determined by: % lysis = 100 × (ER – SR)/(MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively.

Ab-dependent cellular phagocytosis assay

MDMs were derived from peripheral blood monocytes using M-CSF (R&D Systems) at 20 μg/ml for 5–7 d. The MDMs were fluorescently labeled with Min-Claret dye (Sigma-Aldrich). CLL cells were fluorescently labeled with PKH-67 (Sigma-Aldrich) and coated with Ab for 1 h at 4˚C. MDM and CLL cells were cocultivated for 30 min at a 1:5 E:T ratio, then colocalization of CLL with MDM was scored using flow cytometry and verified using microscopy.

Lipid rafts and immunoblot analysis

Lipid raft fractions were isolated using a sucrose gradient, as described previously (41). Purity of lipid raft fractions was determined using cholera-toxin presence. Whole cell extracts were prepared as previously described by our group (42) with the addition of phosphatase inhibitor mixture 1 and 2, protease inhibitor mixture P8340 and 1 mM PMSF (all from Sigma-Aldrich) to the lysis buffer. Equivalent amounts of protein were separated on polyacrylamide gels and transferred onto nitrocellulose membranes. After Ab incubations, proteins were detected with chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). The following Abs were used for detection: anti-phospho-ERK1/2 (Thr202/Y204) and anti-ERK1/2 (Cell Signaling Technologies, Danvers, MA); and anti-pan phosphor-tyrosine Ab 4G10 and anti-GAPDH (Millipore, Billerica, MA), cholera-toxin (Sigma-Aldrich), and actin (Santa Cruz, Santa Cruz, CA). The FcyRIIA and FcyRIIB Abs have been described previously (43).

Statistics methods

Considering each of the patient’s cells (CLL B cells, monocytes, and MDMs) were used under all indicated conditions of each experiment, linear mixed effect models were used to estimate unrestricted covariance structures and robust hypothesis tests (44).

Results

GA101 mediates superior direct cytotoxicity with or without anti-Fc cross-linking Ab

Direct signaling to apoptosis has been shown to have a role in anti-CD20 therapy (19, 45). GA101, as a type II Ab, promotes ~25% cell death of CLL B cells, with a range of 0–61%, in the absence of an Fc cross-linker. This is significantly more than the type I Abs ofatumumab or rituximab in the absence of a cross-linking Ab (Fig. 1A; p = 0.0012 or 0.013, respectively). This direct cytotoxicity study was extended to compare the three anti-CD20 Abs with the Fc cross-linking Ab in a larger sample of patients (n = 19; Fig. 1B). No enhanced cytotoxicity was observed with GA101 treatment when treated with an Fc cross-linking Ab. GA101 mediated significantly greater cytotoxicity with an Fc cross-linking Ab than rituximab (p = 0.003) or ofatumumab (p = 0.03; Fig. 1B). The anti-CD52 Ab alemtuzumab is used in this study, as well as other experiments, as a positive control for Ab function in the assay. Collectively, these studies suggest that type II Ab GA101 mediates superior direct cytotoxicity without requirement for an Fc cross-linking compared with the type I Abs. However, ofatumumab mediated greater direct cytotoxicity with the Fc cross-linking Ab than did rituximab (p = 0.0687).

Ofatumumab mediates superior complement-dependent cytotoxicity

Initiation of complement can be a potent method of cytotoxicity for therapeutic Abs (46) (47). To test the ability of the three CD20-directed Abs to initiate complement, we examined CLL cells for sensitivity to complement-mediated killing. As shown in Fig. 1C, ofatumumab demonstrates ~30% CDC in CLL cells, significantly greater than either rituximab or GA101 (p = 0.0001). This enhanced CDC with ofatumumab was maintained regardless of whether fresh or frozen primary CLL samples were used as targets (data not shown). Quantification of CD20 Ag on CLL patient B cells showed a wide range of CD20 surface expression (17,000–600,000; Fig. 1D) and a weak correlation (r² = 0.3; p = 0.026) between the level of CDC induced by ofatumumab and CD20 surface expression (Fig. 1E). There was no correlation between surface CD20 and CDC seen with rituximab (p = 0.96) or with GA101 (p = 0.836). Collectively, these data demonstrate that ofatumumab is superior to GA101 or rituximab in mediating CDC against CLL cells.

GA101 stimulates enhanced NK cell activation compared with ofatumumab or rituximab

NK cells are the major effector cell population implicated in mediating ADCC. ADCC is initiated through FcγRIIA (CD16) engagement (48) and engineered Abs that are either mutated or lack a core fucosylation in the Fc region generally have enhanced affinity for this receptor. As an afucosylated Ab, GA101 has been reported to mediate enhanced NK cell ADCC (32, 34–36). To compare GA101 to the other anti-CD20 Abs in terms of NK cell function, we first examined NK cell activation by looking at the activation marker CD107a (39). Normal donor NK cells were stimulated with immobilized Ab, and CD107a expression was measured using flow cytometry. As shown in Fig. 2A, GA101 significantly induced more CD107a expression on NK cells than does ofatumumab (p = 0.09) or rituximab (p = 0.005). Furthermore, GA101 promoted a significant amount of IFN-γ release from stimulated normal donor NK cells compared with ofatumumab and rituximab (p = 0.0008; Fig. 2B). There was no significant difference observed in IFN-γ production between ofatumumab and rituximab. Collectively, these data suggest that GA101 is a better activator of NK cells compared ofatumumab or rituximab.

Binding of FcγRs on immune cells and recruitment of these receptors into lipid rafts is necessary for signaling within effector cells. Therefore, we next evaluated the recruitment of FcγRIII to lipid rafts on NK cells after stimulation with immobilized anti-CD20 Ab. To obtain sufficient cells to isolate lipid rafts, we used the NK92 cell line that has been stably transfected to express FcγRIII (39). These cells were stimulated briefly with immobilized Abs, and lipid raft fractions were isolated with subsequent assessment by immunoblot to determine whether FcγRIII was differentially recruited into the rafts. As seen in the representative blot in Fig. 2C, FcγRIII was recruited equally into the raft fraction with GA101 and with ofatumumab. Rituximab showed recruitment to a lesser degree. Analysis of the lipid raft with cholera-toxin revealed the presence of GM1 ganglioside binding activity in the raft, but not in the non-raft fractions, demonstrating purity of the lipid raft preparations used in these studies.

GA101 mediates enhanced NK cell ADCC compared with ofatumumab and rituximab

Activation of NK cells, as measured by CD107a and IFN-γ production, and recruitment of FcγRIII to lipid rafts often correlates with ADCC potential (49). To confirm superiority of GA101 in NK cell function, normal donor NK cells were used as effectors against CLL B cell targets in standard 51Cr-release assays. As expected with an afucosylated Fc region engineered Ab, GA101 mediated significantly more ADCC than ofatumumab (p < 0.0001) or rituximab (p < 0.0001) at 5 μg/ml of Ab (Fig. 2D), respectively.

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However, at decreasing concentrations of Ab, the enhanced ADCC seen with GA101 is no longer apparent. At concentrations of 0.05 \( \mu \text{g/ml} \) and less, GA101 is no longer significantly more effective than ofatumumab (\( p = 0.9761 \)). In addition, at those lower Ab concentrations, ofatumumab is superior in ADCC function compared with rituximab (\( p < 0.0001 \)).

NK cells from CLL patients have been reported to have decreased effector function (50) and are most relevant to the studies pursued in this article. To test these engineered Abs with NK cells from CLL patients, ADCC assays were performed using NK cells from 12 patients with early-stage disease as effectors against Raji cell targets (Fig. 2E). At 10 \( \mu \text{g/ml} \) concentrations GA101, ofatumumab, and rituximab demonstrate insignificant difference in ADCC between NK cells from CLL patients and normal donor NK cells (\( p = 0.141, 0.464, \) and 0.085, respectively), although the CLL cells have an overall trend toward decreased function. Collectively, these data support the findings of others that CLL patients NK cells could have a modest defect in NK cell ADCC (51, 52) against CD20-targeted cells; however, GA101 remains superior to ofatumumab or rituximab at NK cell–mediated ADCC.

**FIGURE 1.** Anti-CD20 Abs differentially mediate direct cytotoxicity and complement dependent cytotoxicity. (A) GA101 mediates significantly greater direct cytotoxicity in CLL cells treated without a cross-linking Ab compared with ofatumumab or rituximab (\( p = 0.0012 \) or 0.013, respectively) at 48 h (\( n = 8 \)). (B) GA101 mediates significantly greater direct cytotoxicity on CLL cells with an Fc cross-linking Ab than rituximab (\( p = 0.003 \)) or ofatumumab (\( p = 0.03 \)). Flow cytometry results after treatment with 10 \( \mu \text{g/ml} \) of each Ab with 50 \( \mu \text{g/ml} \) of goat anti-human anti-Fc cross-linking Ab for 48 h (\( n = 19 \)). (C) Ofatumumab mediates enhanced complement dependent cytotoxicity (CDC) with human serum on previously frozen CLL B cells (\( p = 0.0001 \) for both comparisons; \( n = 19 \)). All Abs were used at 10 \( \mu \text{g/ml} \). (D) Quantification of CD20 on the surface of CLL cells by flow cytometry (\( n = 19 \)). (E) Ofatumumab shows a weak correlation between surface CD20 density and CDC (\( r^2 = 0.3; p = 0.026 \)). *denotes statistical significance (\( p \leq 0.05 \)) between the indicated groups.

**Anti-CD20 Abs differentially stimulate monocytes**

Monocytes have been implicated as being the most important effector cell in CD20-directed Ab efficacy in murine models (6, 8). Similar to the Ab effector function of NK cells, monocyte and MDM activation and phagocytosis is governed by the interplay among FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa, which are all expressed in these cells (53) (54). To compare GA101 and ofatumumab to rituximab in human monocytes and MDM, we tested production of TNF-\( \alpha \) cytokine by monocytes from normal donors. Monocytes were stimulated with immobilized GA101, ofatumumab, or rituximab, and cells or supernatants were collected for mRNA and TNF-\( \alpha \) analysis, respectively. As seen in Fig. 3A, monocytes have decreased mRNA levels of TNF-\( \alpha \) after stimulation with GA101 compared with rituximab or ofatumumab (\( p = 0.0018 \) and \( p < 0.0001 \), respectively). In addition, significantly less TNF-\( \alpha \) is
produced with GA101 compared with rituximab ($p = 0.018$; Fig. 3B). GA101 also produces less TNF-α than ofatumumab, although this difference is not significant ($p = 0.35$; Fig. 3B).

**GA101 demonstrates inferior Ab-dependent cellular phagocytosis compared with ofatumumab or rituximab**

MDMs have also emerged as especially important in anti-CD20 Ab clearance of B cells, particularly in murine models (6). These macrophages have been demonstrated to phagocytose Ab-coated cells predominantly through FcγRIIa, with some contribution from FcγRI and FcγRIIIa (48, 55). We first examined the ability of immobilized GA101, ofatumumab, or rituximab to activate MDM, generated from normal human peripheral blood monocytes, as measured by TNF-α production. MDM cells exposed to plate-bound ofatumumab or rituximab produced higher levels of TNF-α, compared with GA101 ($p = 0.7$ and 0.34, respectively; Fig. 3C). These data support the finding that monocytes and MDM cells demonstrate inferior activation with GA101 compared with ofatumumab or rituximab.

We next examined the ability of GA101, ofatumumab, or rituximab to mediate Ab-dependent cellular phagocytosis (ADCP) by MDMs. Phagocytosis was tested by coincubation experiments of membrane-dyed MDM with Ab-coated, membrane-dyed CLL cells. The cells were analyzed with flow cytometry, and percent phagocytosis was determined by double positivity for both dyes, indicating MDM that had ingested CLL cells. The results were validated with microscopy. Contrary to the TNF-α data, all three of the anti-CD20 Abs showed ADCP capability against CLL B cells (Fig. 3D), with ofatumumab exhibiting the greatest ADCP (60 ± 8.8%) compared with rituximab (48 ± 17.8%) and GA101 (41 ± 16.7%). Ofatumumab was able to mediate significantly more ADCP compared with GA101 ($p = 0.0036$) or rituximab ($p = 0.03$).

Collectively, these data demonstrate that non–FcγR-engineered ofatumumab and rituximab mediate superior MDM activation and phagocytosis compared with GA101.

**Anti-CD20 Abs differentially signal in monocytes**

We next sought to investigate the mechanism of differential TNF-α release and phagocytosis by the CD20-directed Abs. Initial studies determined whether there was differential global tyrosine protein phosphorylation as measured by 4G10 immunoblots from monocytes stimulated with immobilized GA101, ofatumumab, or rituximab for 5–7 min (results shown are representative of three independent experiments). Cholera-toxin blot on the right side indicates purity of raft versus nonraft fractions. (D) ADCP with normal NK cell effectors against CLL B cell targets in decreasing Ab concentrations ($n = 4$ CLL B cell targets with 3 NK cell effectors each). GA101 shows enhanced ADCP at higher concentrations compared with ofatumumab or rituximab ($p < 0.0001$). (E) ADCP with CLL or normal NK cell effectors against Raji cell targets ($n = 12$) shows insignificant difference in ADCP between NK cells from CLL patients and normal donor NK cells with anti-CD20 Abs ($p = 0.141, 0.464, and 0.085$, for GA101, ofatumumab, and rituximab respectively), although the CLL cells have an overall trend toward decreased function. *denotes statistical significance ($p = 0.05$) between the indicated groups.

NR, Nonraft; R, raft.
mediated, we examined for differential phosphorylation and recruitment to lipid rafts where activation signaling is initiated. The activating Fc receptor region is responsible for binding to FcγR and ultimately recruitment to lipid rafts where activation signaling is mediated, we examined for differential phosphorylation and recruitment of FcγRIIA to lipid rafts by immobilized GA101, ofatumumab, and rituximab. The phosphorylation pattern of both the activating FcγRIIa (Fig. 4B) and inhibitory FcγRIIB (Fig. 4C) in monocytes exposed to the different immobilized CD20 Abs did not clarify the differential TNF-α release.

Phosphorylation of the receptor did not explain the differential activation of monocytes; therefore, we hypothesized potential differences in recruitment of the Fcγ receptors into signaling lipid rafts. In Fig. 4D, we demonstrate that GA101 does not induce recruitment of the activating FcγRIIa to lipid rafts in the THP-1 cell line. This finding is in contrast to what is observed with the positive control (IgG), ofatumumab, and rituximab where robust recruitment of this ITAM-containing FcγR occurs. Analysis of the lipid raft with cholera-toxin revealed the presence of GM1-ganglioside binding activity only in the raft, demonstrating purity of the lipid raft preparations (Fig. 4D, left panel).

Next, to determine whether other receptors such as FcγRIIIa and FcγRI were differentially recruited, we investigated the effect of CD20-directed Abs on γ-chain recruitment to lipid rafts (Fig. 4E). Whereas FcγRIIIa contains its own cytosolic ITAM motif, the other activating Fcγ receptors, namely FcγRIIa and FcγRI, associate with an ITAM-containing common γ-chain to initiate activating signals. There were no differences in recruitment of the common γ-chain between the three anti-CD20 Abs tested, indicating no differences in FcγRIIa and FcγRI recruitment. Therefore, GA101 lacks recruitment of only FcγRIIa into lipid rafts in monocytes.

To further explore whether downstream signaling is differentially diminished in GA101-stimulated monocytes, we examined alteration of phospho-ERK following exposure to immobilized Abs. Downstream phosphorylation of ERK following FcγR cross-linking has been demonstrated by others to be critical for the induction of transcription factors such as NF-kB and c-fos and subsequent expression of cytokines (56) (57). In Fig. 4F, we demonstrate that GA101 results in decreased phosphorylation of ERK (T202/Y204), compared with ofatumumab or rituximab in THP-1 cells; this was verified in primary monocytes (data not shown). These results collectively suggest that GA101 demonstrates diminished recruitment of FcγRIIa to lipid rafts and decreased signaling in monocytes compared with ofatumumab or rituximab.

Combination of anti-CD20 Abs with TLR-agonist R-848 enhances MDM cytokine production

TLR 7/8 agonists work predominantly through cytokine production (58). R-848, a TLR 7/8 agonist, has also been shown to increase expression of the activating Fc receptors FcγRI and FcγRIIa, as well as the common γ-chain. Furthermore, monocytes treated with R-848 decreased expression of the inhibitory receptor, FcγRIIB (59). Given this positive link between the TLR7/8 and FcγR pathways, we set out to determine whether the cytokine deficiency seen with the anti-CD20 Abs could be enhanced with R-848. Monocytes or MDM were pretreated overnight with R-848 and subsequently stimulated for 24 h with immobilized Abs. TNF-α levels in the supernatant were measured with ELISA. Monocytes treated with R-848 followed by stimulation with GA101 showed no increase in TNF-α production, similar to R-848 alone stimulated cells (p = 0.92; Fig. 5A). In contrast, MDM pretreated with R-848 showed significantly increased TNF-α production by GA101-stimulated MDM over R-848 alone stimulation (p = 0.0071; Fig. 5B) to levels that were similar to the other anti-CD20 Abs. This finding suggests that combination therapy of GA101 with R-848 can rescue the decreased cytokine release phenotype seen from MDM.

Discussion

In this study, we have shown the divergent effector properties of three clinically relevant anti-CD20 Abs. Both GA101 and ofatumumab are superior to rituximab in separate ways. GA101 displays superior direct cytotoxicity without an in vitro cross-linking Ab. Its engineered Fc region elicits enhanced NK cell stimulation...
FIGURE 4. Anti-CD20 Abs signal differentially in monocytes. (A) Immobilized GA101 induces decreased pan–tyrosine-phosphorylated proteins response in THP-1 cells compared with ofatumumab or rituximab (n = 4). (B) There is no differential phosphorylation of FcγRIIb after stimulation with GA101 and ofatumumab. Primary monocytes stimulated 5–7 min on Ab-coated plates and immunoblots tested for FcγRIIb (n = 3). (C) There is no differential phosphorylation of FcγRIIIa after stimulation with GA101 and ofatumumab. Pan–phospho-tyrosine protein immunoprecipitation performed on primary monocytes stimulated with plated Abs for 5–7 min and immunoblots tested for FcγRIIIa (n = 3). (D) FcγRIIa is not recruited to lipid rafts after stimulating THP-1 cells with immobilized GA101 (representative of three independent experiments). FcγRIla recruitment is seen with ofatumumab and rituximab. Cholera-toxin blot on the right panel indicates purity of raft versus nonraft fractions. (E) The common γ-chain is not differentially recruited to lipid rafts in the same samples as in D, indicating no differential recruitment of FcγRI or FcγRIII (representative of three independent experiments) in THP-1 cells with the anti-CD20 Abs. (F) Immunoblot analysis shows decreased phosphorylation of ERK in THP-1 cells after stimulation with immobilized GA101, compared with ofatumumab and rituximab (results shown are representative of three independent experiments). NR, Nonraft; R, raft.
and IFN-γ release, and subsequently superior NK cell–mediated ADCC. Conversely, ofatumumab exhibits superior complement activation against primary CLL cells, independent of CD20 Ag density; it mediates the greatest MDM-mediated ADCC. In addition, rituximab and ofatumumab elicited TNF-α release from both monocytes and MDM, whereas GA101 exhibited decreased response. Although high levels of TNF-α production after therapeutic Ab infusion might not be clinically desirable (60–62), TNF-α release from mononuclear cells is needed for death signal to target cells and for crosstalk to NK cells (9, 10). In addition, although GA101 was not able to elicit cytokine response from MDM, it triggered moderate ADCP function. This finding suggests that separate pathways are involved for these two functions. Lastly, monocytes showed an overall decrease in tyrosine-phosphorylated proteins after stimulation with GA101 and decreased recruitment of FcγRIIa into lipid rafts.

The effect of Fc region afucosylation of Abs on monocyte function has only been implied by studies looking at the binding affinity of these Abs to FcγRII. Low-fucosylated Abs have moderate to no enhanced binding to FcγRIIa, FcγRIIB, or FcγRI compared with their highly fucosylated counterparts (63, 64). This finding would imply that monocytes, with their predominant expression of FcγRIIa and FcγRIIb, should not be differentially affected by glycoengineered Abs. However, these systems only examine Fc region binding affinities to receptors and not how the receptors interact with the Abs (i.e., recruitment to rafts and signaling). This process has not been described with Fc-engineered Abs. Our results suggest that perhaps there are differences in interaction with alternative FcRs because of glycoengineering.

Lack of recruitment of FcγRIIa into lipid rafts has numerous implications for signaling in monocytes. FcγRIIa has a cytosolic ITAM motif, a sequence of conserved amino acids found on many immune receptors that contains a tyrosine that can be phosphorylated by activating kinases. Unlike GA101, the non–Fc-engineered anti-CD20 Abs are able to recruit FcγRIIa to the rafts, indicating that perhaps Fc engineering for enhanced binding to FcγRII could affect IgG interactions with alternative FcγRs. This lack of recruitment to the rafts after GA101 treatment leads to decreased activation as reflected by a decrease in pan-tyrosine phosphorylated proteins. This finding is further confirmed by diminished phosphorylation of ERK, a downstream target of FcγRIIa signaling, and functionally by decreased cytokine production.

Decreased activation of monocytes and MDM by GA101 could have clinical implications for therapy. Given the role of these cell types in anti-CD20–mediated B cell depletion in mouse models, this could explain the potential diminished efficacy seen with GA101 clinically in CLL and NHL compared with trials done with other CD20-directed Abs (38, 65–66). GA101 showed lymphocyte depletion until week 25, but disease progression in patients with high tumor burden (38). Furthermore, our studies show that NK cell–mediated ADCC is not maintained at a superior level with low Ab concentrations, making dosing schedules highly relevant. However, the deficiencies in monocyte and MDM cytokine production by GA101 can be overcome using combination therapy with TLR 7/8 agonists such as R-848. TLR 7 or 7/8 agonists have been used successfully in the clinic (67) and have been shown to have antitumor responses in murine models (68).

The interaction of FcγRs on effector cells with engineered Fc portions is vital in Ab therapeutics. Although engineered Abs are designed to be effective activators of CLL NK cells in vitro (51), the in vivo role of these Abs in the activation of NK cells, monocytes, and MDM is controversial. Differential effects of the novel CD20 directed Abs reported in this study underline the importance in choosing the ideal CD20 Ab as a single agent or in combination in the context of the functional competency of the relevant effector cell populations in CLL and other B cell malignancies.

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Disclosures

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