γδ T-cell killing of primary follicular lymphoma cells is dramatically potentiated by GA101, a type II glycoengineered anti-CD20 monoclonal antibody

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ABSTRACT

Background
Anti-CD20 monoclonal antibodies are major therapeutic agents for patients with follicular lymphoma and work through complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity. Optimization of antibody-dependent cellular cytotoxicity, in particular by amplifying its effectors, could further increase the efficacy of anti-CD20 monoclonal antibodies.

Design and Methods
We investigated the cytotoxic activity of Vγ9Vδ2 T cells against follicular lymphoma cells and whether this killing could be increased by promoting antibody-dependent cellular cytotoxicity with anti-CD20 monoclonal antibodies, in particular a type-II glycoengineered anti-CD20. Vγ9Vδ2 T cells were expanded *in vitro* in the presence of bromohydrin pyrophosphate (Phosphostim) and interleukin-2 and their ability to kill follicular lymphoma primary cells or cell lines was evaluated by flow cytometry cytotoxic T-lymphocyte assays in the presence or absence of three anti-CD20 monoclonal antibodies: the afucosylated GA101, the chimeric rituximab or the humanized ofatumumab. The ability of these cells to release perforin/granzyme and secrete interferon-γ when co-cultured with follicular lymphoma primary cells or cell lines in the presence or not of the three anti-CD20 monoclonal antibodies was also evaluated by CD107a staining and Elispot assays.

Results
Phosphostim and interleukin-2 expanded Vγ9Vδ2 T cells were cytotoxic to primary follicular lymphoma cells and their cytotoxic potential was dramatically increased by GA101, a type II glycoengineered anti-CD20 monoclonal antibody, and to a lesser extent, by rituximab and ofatumumab. The increased cytotoxicity was associated with increased secretion of perforin/granzyme and interferon-γ.

Conclusions
In-vitro expanded Vγ9Vδ2 T cells efficiently kill primary follicular lymphoma cells and express CD16; anti-CD20 monoclonal antibodies, in particular GA101, dramatically increase the cytotoxic activity of expanded Vγ9Vδ2 T cells. These preclinical results prompt the development of clinical trials using this antibody dependent cellular cytotoxicity property of Vγ9Vδ2 T cells and anti-CD20 monoclonal antibodies.

Key words: γδ T-Lymphocytes, follicular lymphoma, anti-CD20 monoclonal antibody, rituximab, ofatumumab.

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Introduction

Therapeutic monoclonal antibodies have dramatically improved the treatment of cancers. These agents have multiple mechanisms of action combining antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity, antibody-dependent phagocytosis, direct cytotoxic activity, and inhibition of receptor signaling.[1] ADCC occurs when cytolytic effector cells express a receptor for the Fc region of IgG (Fcγ receptors), such as CD16 (FcγRIIIA), making it possible for these effectors to bind target cells labeled by therapeutic monoclonal antibodies. Rituximab, a chimeric anti-CD20 monoclonal antibody is the successful prototypic monoclonal antibody for B-lymphoid malignancies.[2,3] There are two types of anti-CD20 monoclonal antibodies: rituximab-like type I monoclonal antibodies, which activate complement and are less efficient at inducing programmed cell death, and type II monoclonal antibodies, which are less active in complement activation but efficiently promote programmed cell death as IgG or F(ab')$_2$ molecules.[4-7] New humanized monoclonal antibodies against CD20 have been generated. Ofatumumab, a type I anti-CD20 monoclonal antibody, is remarkably efficient at activating complement-dependent cytotoxicity and is able to kill chronic lymphocytic leukemia cells, which express CD20 weakly, in the presence of human plasma or unfractionated peripheral blood.[8-11] GA101, the first type II glycoengineered humanized anti-CD20 IgG1 monoclonal antibody, has been engineered to enhance ADCC through an afucosylated Fc region.[12] It works independently of complement-dependent cytotoxicity mainly by promoting effector cell activation and direct induction of programmed cell death.[13,14]

Several lines of evidence suggest that enhancing ADCC induced by therapeutic monoclonal antibodies may directly improve the clinical efficacy of these antibodies.[15] Firstly, in mice bearing xenografted human tumors, the efficacy of rituximab, ofatumumab, and GA101 relies on the cell-surface expression of FcγR.[8-10,13,16] Secondly, the clinical efficacy of rituximab in patients with B-cell lymphoma is linked to FcγRIIIA polymorphism and thus, to the affinity of rituximab for FcγRIIIA.[17,18] Thirdly, optimizing the affinity of rituximab for FcγRIIIA increased its ADCC and efficacy in preclinical and clinical studies.[19,20] In addition, coating tumor cells with monoclonal antibodies with an enhanced affinity for FcγRIIIA can increase their ability to activate natural killer (NK) cells irrespectively of CD16 polymorphism.[21] Finally, recruitment and activation of additional cell effectors for ADCC might enhance the cytolytic activity of anticancer monoclonal antibodies.[22-25]

The cytolytic effector cells involved in ADCC are CD16$^+$ (FcγRIIIA) NK cells and additional CD8$^+$ cytolytic T-lymphocytes, which release perforin through immunological synapses to kill target cells.[26,27] Human CD4$^-$CD8$^-$ γδ T cells from peripheral blood might also provide an important reservoir of ADCC cytolytic effectors. In humans and non-human primate species, the majority of circulating γδ T-lymphocytes express the Vγ9 T-cell receptor with CD4$^-$ CD8$^-$ TCRVγ9$^+$ cells accounting for 1% to 3% of peripheral blood mononuclear cells. γδ T-lymphocytes are stimulated by non-peptide phosphoantigens, which are small, phosphorylated metabolites produced by the cholesterol pathway in microbial pathogens and tumor cells, and can be highly expanded by phosphoantigens and interleukin-2 (IL-2) *in vitro*. Synthetic analogs, such as bromohydrin pyrophosphate (BrHPP), may mimic natural phosphoantigens and selectively stimulate TCRVγ9$^+$ γδ T-lymphocytes.[28] Phosphoantigen-stimulated γδ T cells proliferate, secrete pro-inflammatory cytokines and chemokines and, most importantly, kill leukemic, multiple myeloma, colon and renal carcinoma cells.[29-34] Several preclinical studies in macaque monkeys[35,36] and clinical studies in cancer patients[31,37-41] have demonstrated the potential of phosphoantigen-activated TCRVγ9+ γδ T-lymphocytes for cancer immunotherapy *in vivo*. Several observations may explain these effects. First, treatment with BrHPP and IL-2 increases the counts of circulating γδ T-lymphocytes 50- to 100-fold, dramatically expanding the reservoir of these effector cells.[42] Secondly, phosphoantigens may drive γδ T-cell maturation from naïve to effector memory cells, some of which express membrane CD16.[43,44] CD16$^+$ effector memory γδ T cells are more efficient than CD16- γδ T cells at binding and killing CD20$^+$ lymphoma cells or other tumor cells.[45-47]

In the current study, we investigated the cytotoxic activity of Vγ9Vδ2 T cells against follicular lymphoma (FL) cells and whether this killing could be increased by promoting ADCC with anti-CD20 monoclonal antibodies, in particular a type-II glycoengineered anti-CD20.

Design and Methods

Collection of peripheral blood samples

Peripheral blood cells were collected by small volume leukapheresis from five patients with FL (median age, 48 years; 2 female and 3 male patients). Patients were included in the study after approval by the institutional review board of the Montpellier University Hospital, France. According to the REAL classification, three patients had grade I FL and two had grade II disease. Buffy coats from eight age-related healthy donors were provided by the French Blood Center (Montpellier, France).

Immunophenotypic analysis

The phenotype of T cells was determined with the following monoclonal antibodies: phycoerythrin (PE)-conjugated CD3, CD4 or CD8, PE-pan-γδ TCR, PE-CD69, PE-CD56, PE-CD16 (Beckman Coulter, Villepinte, France), PE-CXCR4, PE-CCR7 (BD Pharmigen™, France), fluorescein isothiocyanate (FITC) pan-γδ TCR, FITC-Vδ2 TCR (Beckman Coulter), FITC-Vδ1 TCR (Thermo Scientific, France). Corresponding irrelevant isotype-matched mouse monoclonal antibodies were used as negative controls. Briefly, the antibodies were added to 0.5×10^6 whole blood cells followed by 30 min incubation at 4°C. After red cell lysis and washing, 3×10^5 total events or 1×10^5 events in the lymphocyte gate were acquired on a FACScan cytometer (Becton Dickinson) and analyzed with CellQuest software. Lymphocyte subsets were assessed by two-color immunofluorescence analysis.

Cell lines and primary follicular lymphoma cells

Raji and Daudi Burkitt's lymphoma cell lines and the SUDHL6 human B-cell lymphoma cell line (derived from a patient with lymphoma with *BCL2* gene rearrangement) were purchased from the American Type Culture Collection (LGC Promochem, Molsheim, France). The cells were cultured at a density of 2×10^5 cells/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, USA).

In addition, primary tumor cells were isolated from human lymph node biopsies collected from FL patients. FL B cells were

purified as the unbound fraction following magnetic cell sorting using CD2 and CD14 depletion (Dynabeads, Invitrogen). The purity of $CD19^+$ B cells was 90% or more and FL tumor B cells ($CD10^+CD20^+$ cells) were quantified using anti-CD10/CD20 labeling.[48]

In vitro expansion of γδ T-lymphocytes with bromohydrin pyrophosphate and interleukin-2

Peripheral blood mononuclear cells were isolated from fresh blood samples of eight healthy donors and five patients with FL using Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech) and cultured at a density of 10^6 cells/mL at 37°C in 5% CO_2 in RPMI 1640 medium and 10% fetal calf serum. BrHPP (3 μM, Phosphostim; Innate Pharma, Marseille, France) and 300 U/mL IL-2 (Proleukin; Chiron, Basel, Switzerland) were added for 14 days. BrHPP was added once at the onset of the culture and half of the culture medium volume was replaced every 3 days with fresh medium containing 300 U/mL IL-2.

CD107a assay

After culturing γδ T-lymphocytes for 4 h with various stimulators, the frequency of degranulating γδ T-lymphocytes was quantified by measuring CD107a expression.[49,50] γδ T-lymphocytes expanded from peripheral blood mononuclear cells from healthy donors or FL patients were co-incubated with Raji, Daudi, SUDHL6, or autologous primary FL cells at different effector to target cell (E:T) ratios (10:1, 2:1 and 1:2). In some experiments, tumor cells were preincubated with graded concentrations (0.001, 0.01, 0.1, 0.2, 1 and 2 μg/mL) of the three anti-CD20 monoclonal antibodies (rituximab, ofatumumab, GA101), and these concentrations of antibodies were further added to the co-cultures. γδ T-lymphocytes were also stimulated with phorbol-12-myristate-13-acetate (PMA) (2.5 μg/mL) and ionomycin (0.5 μg/mL) (Sigma) for 4 h as a positive control. The PE-conjugated anti-human CD107a monoclonal antibody (BD Bioscience, France) and monensin (9 μM) (Golgi-Stop, BD Biosciences) were added 1 h after starting the cultures. At the end of culture, cells were washed twice and labeled with a FITC anti-pan-γδ TCR monoclonal antibody (Beckman Coulter, France) and $CD107a^+$ γδ T-lymphocytes determined using a FACScan device.

Flow cytometric cytotoxic T-lymphocyte assay

The cytotoxic potential of expanded γδ T cells against allogenic or autologous tumor cells was assayed using the CyToxiLux^{R}Plus! Kit Easy (OncoImmunin, Gaithersburg, MD, USA) with different E:T ratios of γδ T-lymphocytes to tumor cells: 10:1, 2:1 and 1:2. In some experiments, tumor cells were preincubated with 1 μg/mL of an anti-CD20 monoclonal antibody (rituximab, ofatumumab, or GA101), which was further added in the co-culture (1 μg/mL). Target cells were labeled with a fluorescent dye and then co-incubated with effector cells in the presence of a fluorogenic caspase substrate according to the manufacturer's recommendations (*Standard Protocol*). After washes, fluorescence was quantified using a FASCan device.

Interferon-γ enzyme-linked immunosorbent spot assay

The frequency of interferon-γ (IFNγ)-producing γδ T-lymphocytes was assayed with a human IFNγ enzyme-linked immunosorbent spot (Elispot) kit according to the manufacturer's recommendations (Diaclone, USA). γδ T-lymphocytes were stimulated with allogenic or autologous tumor cells for 24 h. The assay was conducted with different E:T ratios of γδ T-lymphocytes to tumor cells: 10:1, 2:1 and 1:2. In some experiments, tumor cells were preincubated with an anti-CD20 monoclonal antibody, which was further added in the co-culture cells (1 μg/mL, rituximab, ofatumumab, or GA101). Phytohemagglutinin (PHA, 3 μg/mL) was used as a known inducer of IFNγ by γδ T-lymphocytes.

Statistical analysis

The significance of the data was evaluated with a Student's parametric test and the Mann-Whitney and Kruskal Wallis non-parametric tests using SPSS 10 software.

Results

Expansion and phenotype of peripheral blood γδ T-lymphocytes from patients with follicular lymphoma or healthy donors in response to bromohydrin pyrophosphate and interleukin-2

Peripheral blood γδ T-lymphocytes from five patients with FL and eight healthy donors were expanded with BrHPP and IL-2 for 14 days. As previously reported for other cancers,[30] peripheral blood γδ T-lymphocytes from patients with FL could be as efficiently expanded as those of healthy donors. The median rate of expansion was 214-fold for FL patients (range, 33-1223), which was not significantly different (P=0.3) from the 80-fold (range, 10-760) expansion for healthy donors. These γδ T-lymphocytes were mainly $CD4^-CD8^-$ γδ2 T-lymphocytes. About 24% of expanded γδ T-lymphocytes expressed CD16, a percentage significantly lower than that in peripheral blood γδ T-lymphocytes of patients with FL (55.6%, P=0.04), but similar to that in circulating γδ T-lymphocytes of healthy donors. Expanded γδ T-lymphocytes from patients with FL and donors similarly expressed the CD69 activation molecule (88.3% and 89.2%, respectively) and CD56 (41.8% and 43.8%, respectively). They did not express CCR7 chemokine receptor and one-third expressed CXCR4 chemokine receptor (Table 1).

GA101 enhanced perforin/granzyme degranulation by γδ T-lymphocytes in the presence of allogeneic or autologous follicular lymphoma cells

Expanded γδ T cells from patients with FL poorly expressed CD107a, a marker associated with the degranulation of cytotoxic T-lymphocytes or NK cells. Unlike Raji cells, Daudi cells efficiently induced CD107a expression in γδ T cells in agreement with the high γδ T-cell activation potential of these latter cells (Figure 1A). SUDHL6 lymphoma cells poorly stimulated CD107a expression by γδ T cells. Similar data were obtained with expanded γδ T cells from healthy donors' peripheral blood (Figure 1B).

We then tested whether pretreatment of primary FL cells by three clinical-grade anti-CD20 monoclonal antibodies - rituximab, ofatumumab, or GA101 - could increase CD107a expression in γδ T cells when stimulated by primary FL cells. Alone, the three anti-CD20 monoclonal antibodies did not increase CD107a expression in γδ T-lymphocytes (*results not shown*). However, pretreatment of primary FL cells with the anti-CD20 monoclonal antibodies significantly (P=0.02) increased CD107a expression in γδ T-lymphocytes at three graded E:T ratios. A significant increase was already detected with a concentration of 0.1 μg/mL of anti-CD20 monoclonal antibody, was maximal with 1 μg/mL, and declined at higher concentrations (Figure 2A-C). The same results were obtained with SUDHL6 lymphoma cells used as stimulators. The 1 μg/mL concentration was used in the subsequent experi-

ments. Similar data were obtained with expanded γδ T-lymphocytes from healthy donors or patients with FL. PMA and ionomycin triggered a maximum of 21% γδ T-lymphocytes to express CD107a and this percentage was unaffected by SUDHL6 lymphoma cell stimulation (Figure 3A). CD107a expression in γδ T-lymphocytes in the presence of SUDHL6 lymphoma cells was increased 5-fold when SUDHL6 lymphoma cells were preincubated with GA101, 3-fold with ofatumumab and 2-fold with rituximab at E:T ratios of 1:2, 2:1 and 10:1 ($P<0.001$) (Figure 3A). Similar data were obtained when primary FL tumor cells were used to stimulate γδ T-lymphocytes from the same patient. Preincubation of autologous tumor cells with GA101 increased CD107a expression by γδ T-lymphocytes by 70% compared to that of γδ T-lymphocytes stimulated by autologous tumor cells alone ($P\leq0.05$, Figure 3B). Thus, pretreatment of FL cells with anti-CD20 monoclonal antibodies could increase the degranulation of patients' γδ T-lymphocytes, the most prominent effects being found with GA101, the first type II glycoengineered monoclonal antibody, and ofatumumab.

γδ *T-lymphocytes efficiently killed autologous primary follicular cells and this killing was potentiated by GA101*

Expanded γδ T-lymphocytes from patients with FL or healthy donors did not readily kill SUDHL6 lymphoma cells. However, pretreatment of SUDHL6 lymphoma cells with the GA101 anti-CD20 monoclonal antibody dramatically increased SUDHL6 cell killing by γδ T-lymphocytes from patients with FL (5-fold increase) at the E:T ratios 10:1, 2:1, or 1:2 ($P<0.05$) (Figure 4A). The same held true for γδ T-lymphocytes from healthy donors (*data not shown*). We demonstrated in this study for the first time that FL patients' γδ T-lymphocytes efficiently killed autologous primary FL tumor cells, and that pretreatment with GA101 increased this killing by 1.6-, 3.9- and 8.4-fold at E:T ratios of 10:1, 2:1 and 1:2, respectively ($P<0.05$) (Figure

Table 1. Phenotype of peripheral blood γδ T-lymphocytes from patients with FL or healthy donors in response to BrHPP and IL-2.

	FL patients (n=5)				Healthy donors (n=8)			
	Day 0		Day 14		Day 0		Day 14	
	Median	Range	Median	Range	Median	Range	Median	Range
CD3 cells (%)	57.0	25.2-68.2	98.5*	96.3-99.5	67.1	59.2-70.7	98.18*	95.2-99.4
γδ T cells (%)	2.9	2.0-7.5	92.6*	85.4-97.2	4.7	1.4-6.8	84.0*	37.2-90
γδ1 Tcells (%)	1.9	0.8-5.2	2.3	0.2-12.3	1.8	1.0-2.9	1.8	0.9-5.2
γδ2 T cells (%)	1.2	0.7-2.8	90.0*	70.6-97.0	3.9	0.6-5.9	77.3*	38.1-89.3
γδT/CD16$^+$ cells (%)	55.6	37.4-69.8	24.4*	2-49.2	27.7•	11-53.7	23.0	7.2-47.5
γδ T/CD69$^+$ cells (%)	12.6	8-18.9	88.3*	67.9-97.5	35.9•	25.5-39	89.2*	68.9-94.3
γδ T/CD56$^+$ cells (%)	35.9	26.3-57.2	41.8	40.3-81.5	28.9	22.4-37.5	43.8	28-52.9
γδ T/CCR7$^+$ cells (%)	1.8	0-38.5	1.0	0.7-4.7	2.8	2.1-4.4	1.6	0.4-5.2
γδT/CXCR4$^+$ cells (%)	61.9	48.6-81.3	35.1*	1.7-49.9	98.8	96.2-99.6	34.9*	19-41.8
CD4 cells (%)	35.9	15.9-38.2	1.9	0.6-4.3	44.4	35.3-53.8	12.1	4.2-39.7
CD8 cells (%)	17.0	3.4-29.3	2.5	1.7-9.6	32.3	23.8-50.4	10.7	0.7-22.1

*Peripheral blood mononuclear cells from five patients with FL and eight healthy donors were cultured with BrHPP and IL-2 as described in Design and Methods section. After 14 days of culture, cells were counted and their phenotype was evaluated by labeling with various monoclonal antibodies (CD3, CD4, CD8, pan γδ T cells, anti-δ1, anti-δ2, CD16, CCR7, CXCR4, CD69 and CD56) and FACS analysis. Data are the median values and the range of the percentage of labeled cells. *The asterisks mean significant differences between day 0 and day 14, the filled circles mean significant differences between healthy donors and FL patients ($P\leq0.05$).*

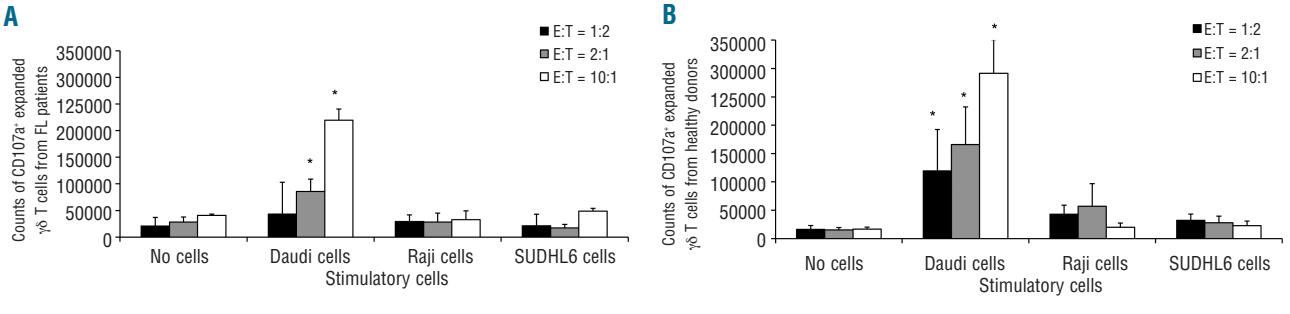

Figure 1. Induction of CD107a expression by expanded γδ T-lymphocytes by allogeneic cell lines. Counts of CD107a$^+$ cells within γδ T-cell population. CD107a$^+$ γδ T-lymphocytes were determined by analyzing at least 10,000 events in the γδ T-lymphocyte gate (10,000 to 20,000 events). The median rate of γδ T-cell expansion was 214 for healthy donors and 80 for FL patients. (A) 10^6 *in vitro* expanded γδ T-lymphocytes (harvested at day 14) from five patients with FL were stimulated for 4 h with various B-cell lines (Raji, Daudi, SUDHL6) at effector:target (E:T) ratios of 10:1, 2:1, or 1:2 and the expression of CD107a by γδ T-lymphocytes was evaluated by FACS. Data are the mean count ± SD of CD107a$^+$ γδ T cells obtained with the five γδ T-lymphocyte preparations. (B) 10^6 *in vitro* expanded γδ T-lymphocytes (harvested at day 14) from six healthy donors were stimulated for 4 h with various B-cell lines (Raji, Daudi, SUDHL6) at E:T ratios of 10:1, 2:1, or 1:2 and the expression of CD107a by γδ T-lymphocytes was evaluated by FACS. Data are the mean count ± SD of CD107a$^+$ γδ T cells obtained with the six γδ T-lymphocyte preparations. *means that the mean value is significantly different from that obtained with unstimulated T-lymphocytes using a t-test for pairs.

4B). GA101 was far more efficient in promoting killing of allogeneic SUDHL6 lymphoma cells than either rituximab (P=0.03) or ofatumumab (P=0.02) (Figure 4A). It was also more efficient in promoting killing of autologous FL tumor cells than either rituximab (P=0.03) or ofatumumab (P=0.02) at E:T ratios of 2:1 and 1:2 (Figure 4B).

GA101 potentiated interferon-γ secretion by γδ T-lymphocytes stimulated by follicular lymphoma cells

Expanded γδ T-lymphocytes from patients with FL or healthy donors secreted IFNγ poorly (60±19 spots/10^4 cells). IFNγ secretion was increased 5-fold by PHA polyclonal stimulation (312±65 spots/10^4 cells, P=0.04) or 3.3-fold by Daudi cells (202±72 spots/10^4 cells, P=0.0003) compared to unstimulated γδ T-lymphocytes. Whereas SUDHL6 lymphoma cells did not increase IFNγ secretion by γδ T-lymphocytes (64±38 spots/10^4 cells), preincubation of these cells with GA101 doubled the secretion (145 spots/10^4 cells, P=0.01) at E:T ratios of 2:1 and 10:1. Pretreatment of SUDHL6 lymphoma cells with rituximab (P<0.05) or ofatumumab (P<0.05) enhanced IFNγ secretion at the same ratios, but less efficiently than GA101 (P<0.05) (Figure 5A). Similar data were obtained with expanded γδ T-lymphocytes from healthy donors (*data not shown*). Stimulation of patients' γδ T-lymphocytes by autologous primary FL tumor cells increased IFNγ secretion by 4-fold (187 spots/10^4 cells *versus* 40/10^4 cells, n=5, P<0.001). In this autologous model, there was a 2.5-fold increase of IFNγ secretion when autologous primary FL tumor cells were preincubated with GA101 at an E:T ratio of 10:1 (P<0.001).

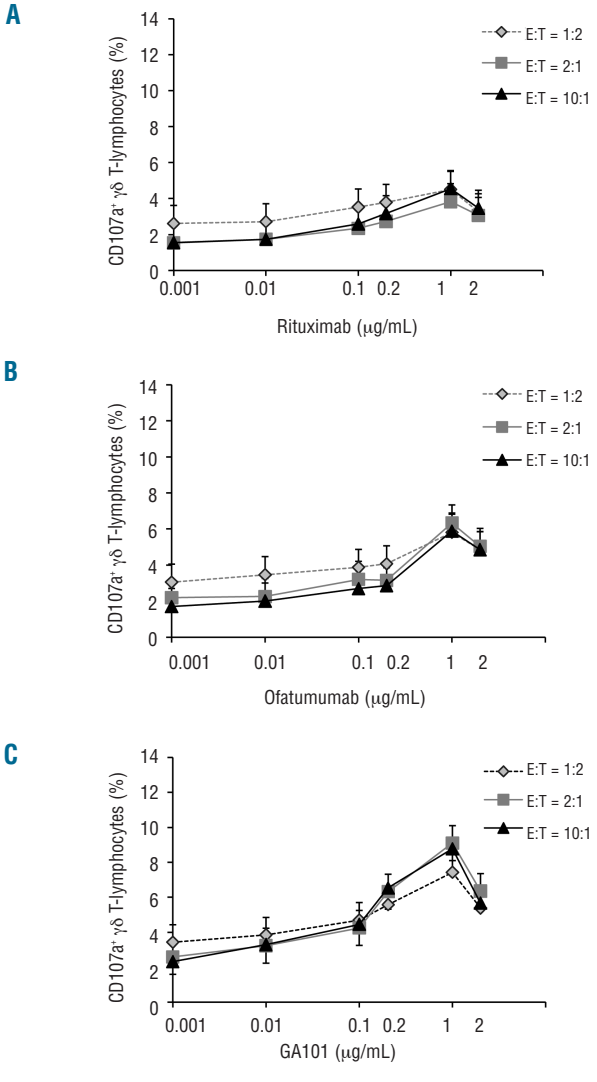

Figure 2. Anti-CD20 monoclonal antibodies increase CD107a expression by γδ T-lymphocytes stimulated by primary FL cells. Expanded γδ T-lymphocytes from six healthy donors were stimulated for 4 h with primary FL cells at different E:T ratios: (10:1, 2:1, 1:2). Primary FL cells were preincubated with different concentrations (0.001, 0.01, 0.1, 0.2, 1 μg/mL) of rituximab (A), ofatumumab (B), or GA101 (C) anti-CD20 monoclonal antibodies and the monoclonal antibodies were further added at the indicated concentration in the 4 h co-culture. Results are the mean percentage of CD107a$^+$ γδ T-lymphocytes from five FL patients.

Discussion

Monoclonal antibody-based therapies to cell surface targets on malignant cells have dramatically improved the

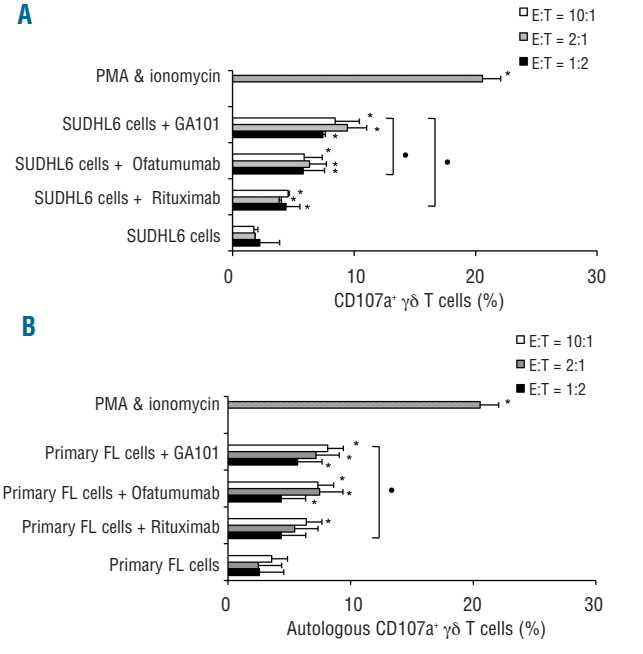

Figure 3. Anti-CD20 monoclonal antibodies increase CD107a expression by FL patients' γδ T-lymphocytes stimulated by autologous FL tumor cells. (A) Expanded γδ T-lymphocytes from five patients with FL were stimulated for 4 h with SUDHL6 lymphoma cells at different E:T ratios (10:1, 2:1, 1:2). SUDHL6 lymphoma cells were preincubated with 1 μg/mL of GA101, ofatumumab, or rituximab and the monoclonal antibodies (1 μg/mL) were further added in the 4 h co-culture. PMA and ionomycin stimulation was used as a positive control to induce CD107a expression. Results are the mean percentages of CD107a$^+$ γδ T-lymphocytes from five patients with FL. (B) Expanded γδ T-lymphocytes from five patients with FL were stimulated for 4 h with purified autologous FL tumor cells at different E:T ratios (10:1, 2:1, 1:2). Autologous FL tumor cells were preincubated with 1 μg/mL of GA101, ofatumumab, or rituximab and the monoclonal antibodies (1 μg/mL) were further added in the 4 h co-culture. PMA and ionomycin stimulation was used as a positive control to induce CD107a expression. Results are the mean percentage of CD107a$^+$ γδ T-lymphocytes from five patients with FL. *means that the mean value is significantly different from that obtained without anti-CD20 monoclonal antibodies using a t test for pairs. •means that the mean value is significantly different between each monoclonal antibody.

treatment of some cancers. However, the responses are often incomplete or not durable and additional therapies with synergistic activities and non-overlapping toxicities will be required to improve clinical outcomes. We have shown here that Vγ9Vδ2 T-lymphocytes with ADCC potential can be expanded *in vitro* with BrHPP and IL-2 stimulation. The expanded Vγ9Vδ2 T cells did not readily kill the allogeneic FL CD20+ SUDHL6 tumor cell line. The killing was, however, dramatically enhanced when the SUDHL6 cells were preincubated with anti-CD20 monoclonal antibodies. This effect was observed with three clinical grade anti-CD20 monoclonal antibodies, the chimeric rituximab and two new monoclonal antibodies derived from the same F(ab')$_2$, the humanized ofatumumab and a first type II glycoengineered monoclonal antibody, GA101. Consistent with the Fc fragment afucosylation of GA101 which enhances binding to Fc receptor,[14,51-53] the most prominent effect was observed with GA101.

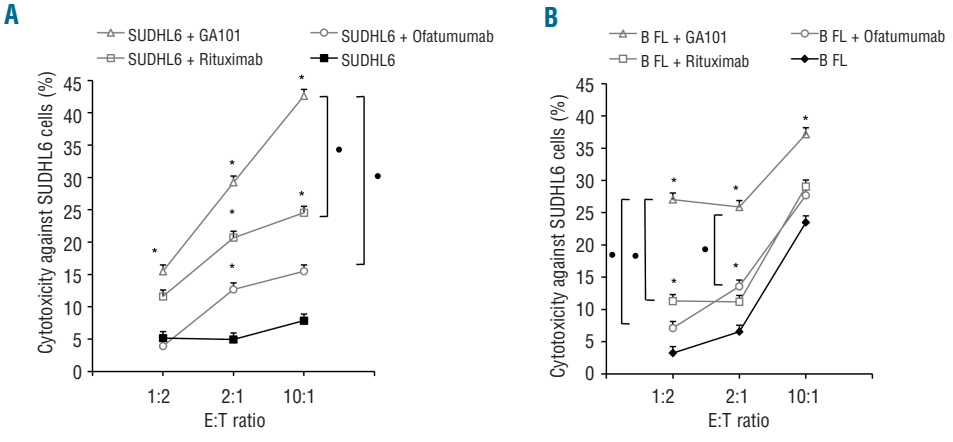

Figure 4. GA101, the first type II glycoengineered anti-CD20 antibody dramatically increases the lysis of allogenic or autologous FL tumor cells by γδ T-lymphocytes. (A) Percentage of lysis of SUDHL6 lymphoma cells by γδ T-lymphocytes at different E:T ratios (10:1, 2:1, or 1:2). In some experiments, SUDHL6 cells were preincubated with 1 μg/mL of GA101, ofatumumab, or rituximab and the monoclonal antibodies were further added in the cytotoxic assay. Data are means of the percentages of lysed SUDHL6 cells with γδ T-lymphocytes from five patients with FL. (B) Percentage of lysis of autologous FL tumor cells by γδ T-lymphocytes at different E:T ratios (10:1, 2:1, or 1:2). In some experiments, autologous FL tumor cells were preincubated with 1 μg/mL of GA101, ofatumumab, or rituximab and the monoclonal antibodies were further added in the cytotoxic assay. Data are means of the percentages of lysed autologous tumor cells with γδ T-lymphocytes from five patients with FL. * means that the mean value is significantly different from that obtained without anti-CD20 monoclonal antibodies using a t test for pairs. • means that the mean value is significantly different between each monoclonal antibody.

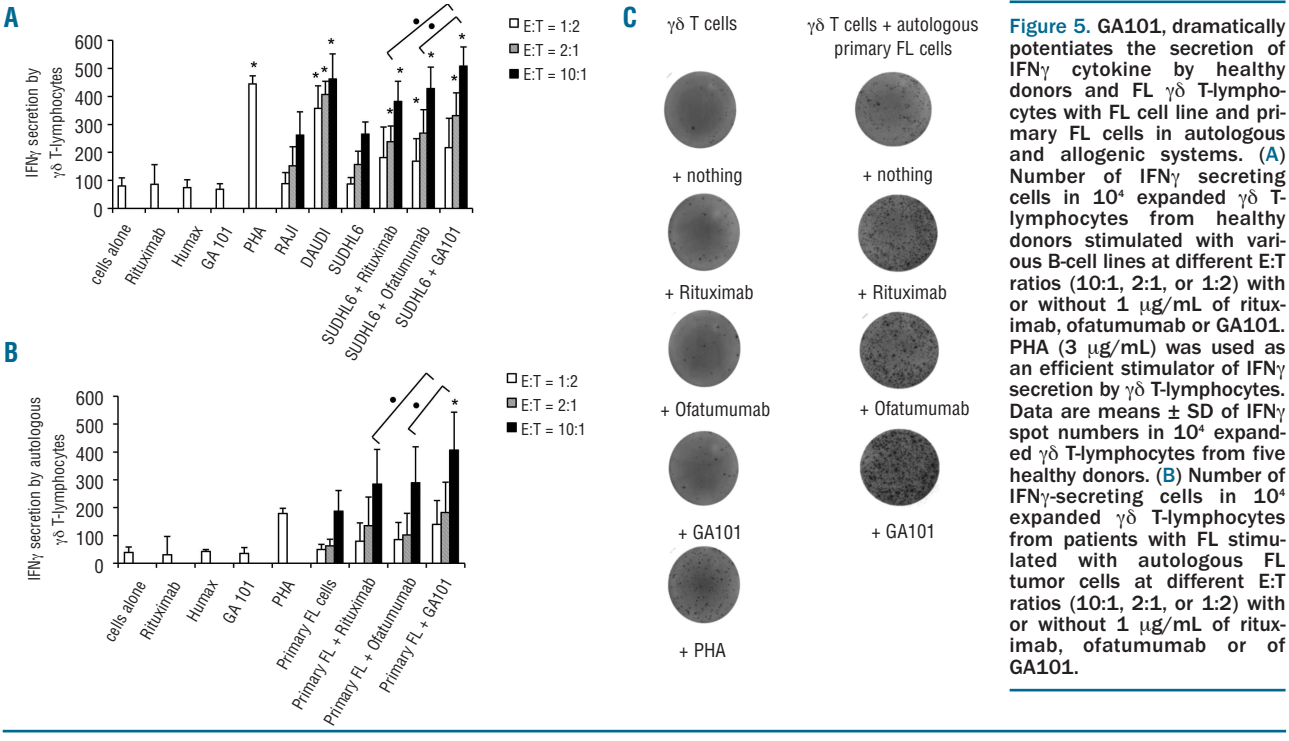

Figure 5. GA101, dramatically potentiates the secretion of IFNγ cytokine by healthy donors and FL γδ T-lymphocytes with FL cell line and primary FL cells in autologous and allogenic systems. (A) Number of IFNγ secreting cells in 10^4 expanded γδ T-lymphocytes from healthy donors stimulated with various B-cell lines at different E:T ratios (10:1, 2:1, or 1:2) with or without 1 μg/mL of rituximab, ofatumumab or GA101. PHA (3 μg/mL) was used as an efficient stimulator of IFNγ secretion by γδ T-lymphocytes. Data are means ± SD of IFNγ spot numbers in 10^4 expanded γδ T-lymphocytes from five healthy donors. (B) Number of IFNγ-secreting cells in 10^4 expanded γδ T-lymphocytes from patients with FL stimulated with autologous FL tumor cells at different E:T ratios (10:1, 2:1, or 1:2) with or without 1 μg/mL of rituximab, ofatumumab or of GA101. PHA (3 μg/mL) was used as an efficient stimulator of IFNγ secretion by γδ T-lymphocytes. Data are means ± SD of IFNγ spot numbers in 10^4 expanded γδ T-lymphocytes from five patients with FL. (C) Illustration of an Elispot assay using γδ T-lymphocytes from one patient with FL stimulated with autologous FL tumor cells with or without 1 μg/mL of rituximab, ofatumumab, or GA101 at an E:T ratio of 10:1. * means that the mean value is significantly different from that obtained without anti-CD20 monoclonal antibodies using a t test for pairs. • means that the mean value is significantly different between each monoclonal antibody.

The increase in cell killing of GA101-labeled SUDHL6 cells by Vγ9Vδ2 T-lymphocytes was associated with an increase in degranulation (CD107a membrane expression) and IFNγ secretion of Vγ9Vδ2 T-lymphocytes. The ability to kill anti-CD20 monoclonal antibody-labeled SUDHL6 cells was observed with Vγ9Vδ2 T-lymphocytes expanded from peripheral blood mononuclear cells from both healthy donors and patients with FL.

A second major and new finding is that expanded Vγ9Vδ2 T-lymphocytes from patients with FL can efficiently kill autologous purified primary tumor cells. This killing was enhanced when primary cells were preincubated with anti-CD20 monoclonal antibodies, the most dramatic increase being obtained with GA101, the type II glycoengineered anti-CD20 monoclonal antibody. Autologous tumor cells also increased CD107a expression by Vγ9Vδ2 T-lymphocytes and CD107a expression was enhanced by adding the three anti-CD20 monoclonal antibodies, especially GA101. Autologous tumor cells also induced IFNγ secretion by Vγ9Vδ2 T-lymphocytes.

Combining Vγ9Vδ2 T-cell adoptive immunotherapy with anti-CD20 monoclonal antibodies,[47,54] in particular type II glycoengineered monoclonal antibodies, could offer an alternative treatment to FL patients refractory to the classical type I monoclonal antibodies. There are several advantages in using Vγ9Vδ2 T-lymphocytes for adoptive immune therapy. First, large numbers of autologous peripheral blood Vγ9Vδ2 T cells can be collected by leukapheresis and cryopreserved. Several billion Vγ9Vδ2 T-lymphocytes can be expanded *ex vivo* with BrhPP and IL-2 starting from one thawed leukapheresis product[30] and the feasibility and safety of administration of these Vγ9Vδ2 T-lymphocytes has already been proven in cancer patients.[31,32] Secondly, tumor-infiltrating lymphocytes contain a high percentage of γδ T cells[55,56] suggesting that adoptively transferred γδ T cells would be able to infiltrate into tumor sites. A phase 1 trial with BrHPP-expanded γδ T lymphocytes has shown that 5-10×10^9 γδ T cells can be obtained starting from one leukapheresis product. As we have shown here that BrHPP and IL-2 can expand γδ T cells from patients with FL as efficiently as from healthy individuals, one could expect to get 5-10×10^9 clinical grade γδ T cells from FL patients starting from one leukapheresis product. The expanded γδ T cells will have to be injected together with GA101 after having already pretreated the patient with GA101.[41]

Thirdly, γδ T-lymphocytes have potential antigen-presenting cell characteristics,[57] providing an additional therapeutic role. This antigen-presenting cell capacity can be increased by the direct binding of Vγ9Vδ2 T cells to monoclonal antibody-labeled tumor cells which may facilitate transfer of antigens from apoptotic tumor cells to Vγ9Vδ2 T cells.[58] In conclusion, we have shown *in vitro* that expanded Vγ9Vδ2 T cells have greater antitumor activity through ADCC when used in combination with tumor-targeting monoclonal antibodies, in particular GA101, the first type II glycoengineered humanized anti-CD20 monoclonal antibody.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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