rILYd4, a Human CD59 Inhibitor Enhances Complement Dependent Cytotoxicity of Ofatumumab against Rituximab Resistant B-cell Lymphoma Cells and Chronic Lymphocytic Leukemia, Ge et al

Supplementary materials and methods for Ge et al.

Antibodies and reagents

RTX was purchased from Biogen Idec (Cambridge, MA). OFA was kindly provided by Genmab (1). Mouse anti-human CD55 and CD46 mAbs (Serotec, Raleigh, NC), mouse anti-human CD20 monoclonal Ab (Abcam, Cambridge, MA), and mouse anti-human CD59 Abs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for flow cytometry analysis. Polyclonal rabbit anti-human C1q Ab was purchased from DakoCytomation (Carpinteria, CA). mAb FITC-1H8, specific for C3b/iC3b/C3dg was kindly provided by Dr. Ronald Taylor (University of Virginia, Charlottesville, VA). Polyclonal goat anti-human C9 Ab was purchased from Advanced Research Technologies(San Diego, CA). The rILYd4 tagged with HisX6 at its N-terminals was purified using the His-bind purification kit (Novagen, San Diego, CA) according to our previously published procedure (2, 3). Removal of endotoxin from the recombinant rILYd4 was accomplished by Detoxi-Gel column (Thermo Scientific, Waltham, MA) to

achieve endotoxin levels that were lower than 0.1 EU/ml as determined by Recombinant Factor C Endotoxin Detection System (Lonza, Walkersville, MD). NHS for CDC assay was purchased from CompTech (Tyler, TX). Alamar Blue reagent was purchased from Serotec (Raleigh, NC). Propidium iodide (PI) was purchased from Sigma-Aldrich (Carlsbad, CA).

Alamar blue assay for measurement of CDC effect

We followed the protocol as we published previously in (4) to perform Alamar blue assay. Briefly, 2 X 10^5 cells per well were seeded on 96-well plates. Different concentrations of RTX or OFA, and NHS were added to the wells (total volume 200 µl/well) in the absence or presence of the different concentrations of rILYd4, and plates were incubated at 37°C for 4 hours. Then 30 µl Alamar blue and 70 µl culture medium were added to each well and incubated overnight. Cytolysis was assessed by reading the plates in an F-2000 fluorescence spectrophotometer (Hitachi, Schaumburg, IL) (excitation: 560 nm; emission: 590 nm). The medium alone (without cells), NHS alone, and heat-inactivated NHS (IHS, 56 ºC, 1 h) alone were used as background controls in the relevant calculation. The positive control was Triton X-100 (0.1% in DPBS) and controls were cells without any treatment. Percent cell death in each well was calculated

as: (fluorescence in untreated control wells – fluorescence in treated well) / fluorescence in untreated control wells X 100.

References:

1. Teeling JL, French RR, Cragg MS, van den BJ, Pluyter M, Huang H, *et al.* Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood 2004;104(6):1793-800.

2. Hu W, Yu Q, Hu N, Byrd D, Amet T, Shikuma C, *et al.* A high-affinity inhibitor of human CD59 enhances complement-mediated virolysis of HIV-1: implications for treatment of HIV-1/AIDS. JImmunol 2010;184(1):359-68.

3. Hu W, Ferris SP, Tweten RK, Wu G, Radaeva S, Gao B, *et al.* Rapid conditional targeted ablation of cells expressing human CD59 in transgenic mice by intermedilysin. NatMed 2008;14(1):98-103.

4. Hu W, Ge X, You T, Xu T, Zhang J, Wu G, *et al.* Human CD59 inhibitor sensitizes rituximab-resistant lymphoma cells to complement-mediated cytolysis. Cancer Res 2011;71:2298-307.

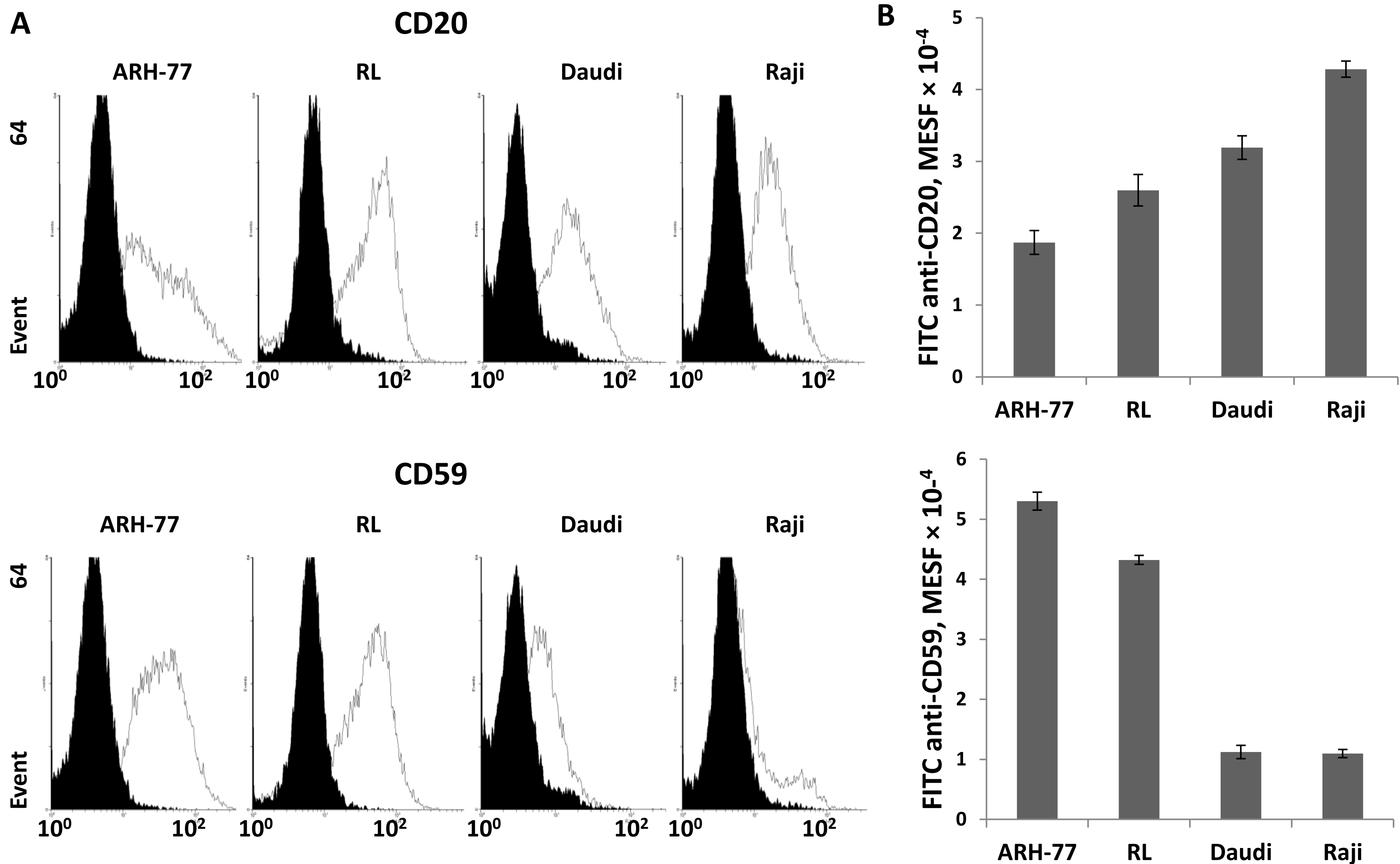


Figure S1: CD20 and CD59 expression level on B-cell malignancy cell lines. (A) FACS analysis of CD20 and CD59 expression levels on ARH-77, RL, Daudi and Raji cells. Black curve: IgG isotype plus secondary antibody conjugated with FITC; white curve: anti-human CD20 or CD59 antibody plus secondary antibody conjugated with FITC. (B) Quantitation analysis of the CD20 and CD59 expression levels. Mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) using calibrated beads. Results are mean ± SD of three different experiments.

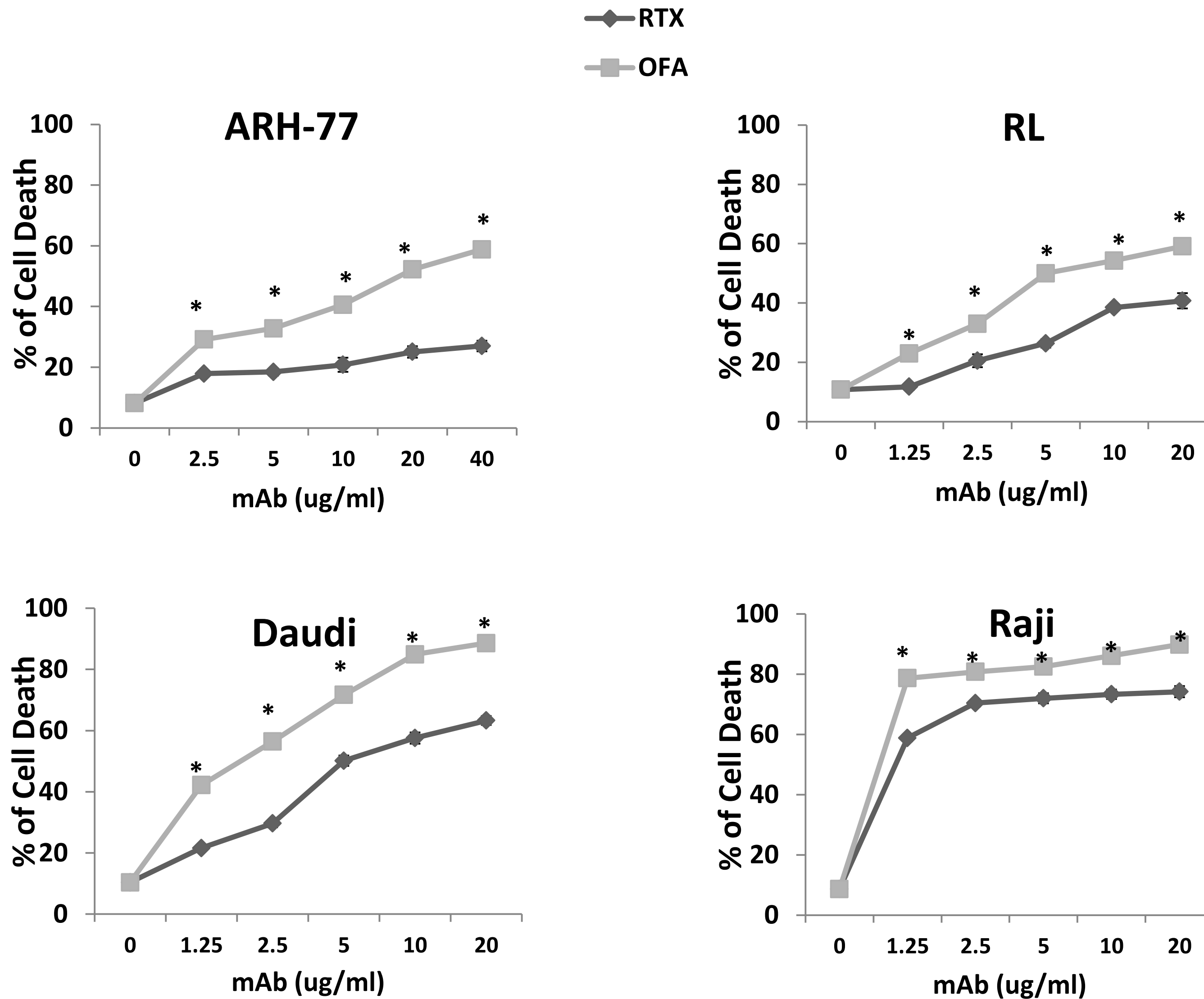
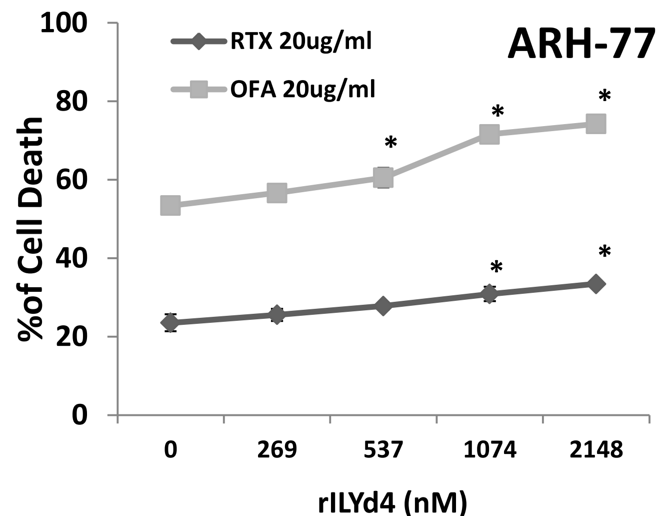
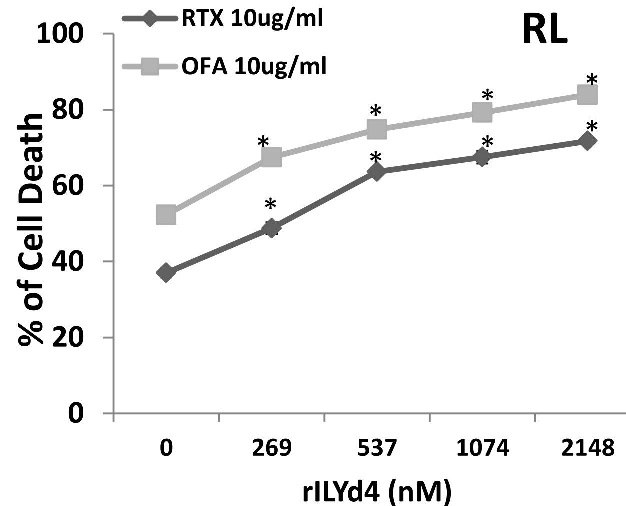
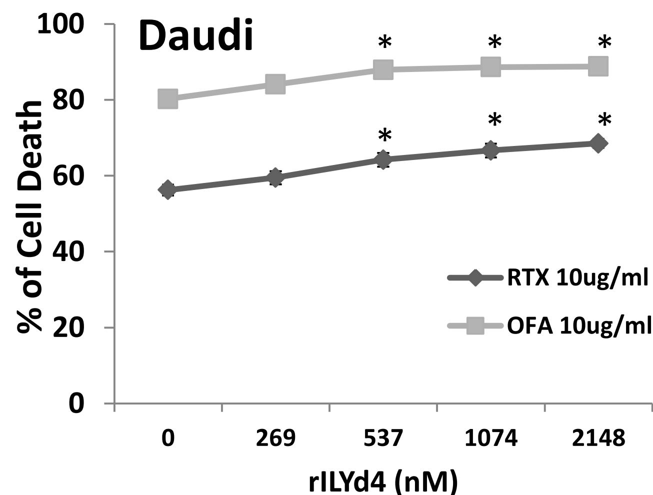


Figure S2: Ofatumumab (OFA) and Rituximab (RTX)-mediated dose dependent complement-dependent cytotoxicity (CDC) effects on the different cell lines. ARH-77, RL, Daudi. Raji were treated with different concentrations of RTX and OFA with 20% normal human serum (NHS) (for ARH-77 and RL) or 5% NHS (for Daudi and Raji). The cell viability was assessed by Alamar blue method. Result are mean $\pm$ SD of three different experiments. *: $P < 0.01$ vs RTX.







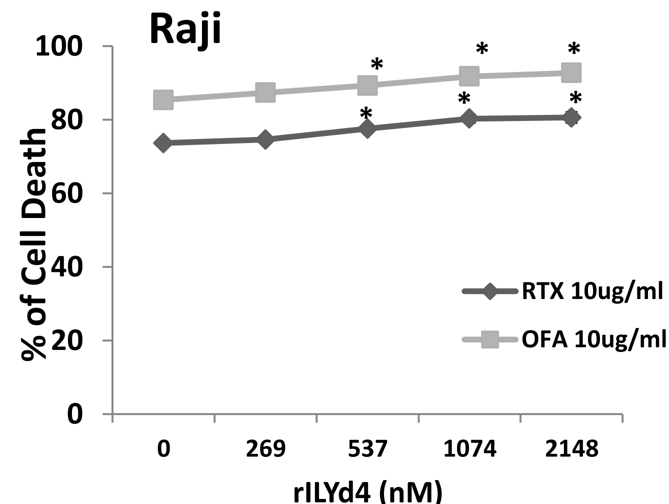


Figure S3: rILYd4 enhances OFA or RTX-mediated CDC effects on the four different cell lines. ARH-77, RL, Daudi, or Raji were treated with different concentrations of rILYd4. 20% or 5% of NHS were used as a source of complement to treat ARH-77 and RL or Daudi and Raji, respectively. Results are mean ± SD of three different experiments. The cell viability was assessed by Alamar blue method. *: $P < 0.01$ vs no rILYd4 treatment.

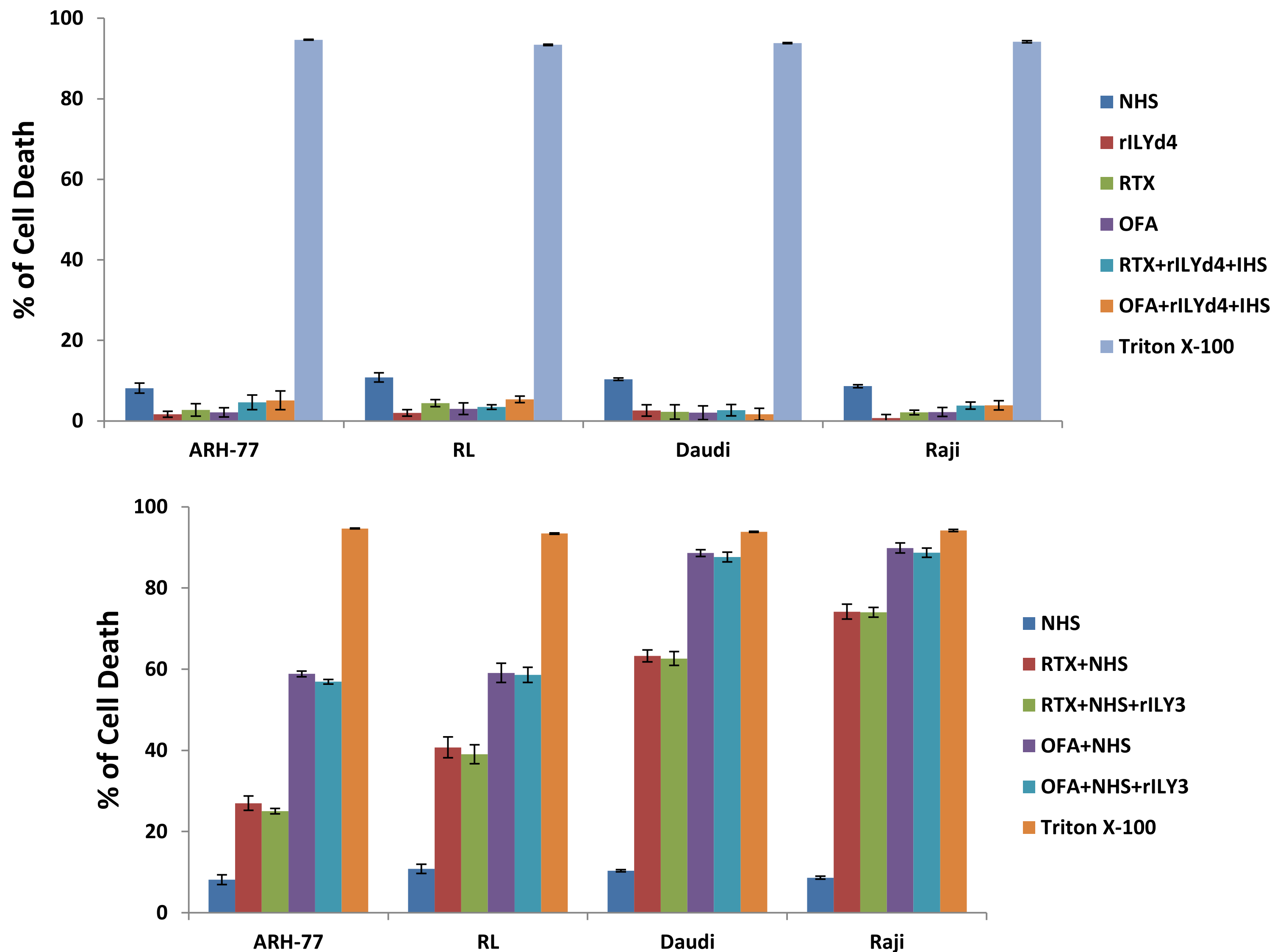


Figure S4: Cell death of ARH-77, RL, Daudi. Raji cells after different treatments. The concentration of rILYd4 was 1074 nM. The concentration of Abs was 20ug/ml for RL, Daudi and Raji, and 40ug/ml for ARH-77. NHS or IHS were 20% for ARH-77 and RL, 5% for Daudi and Raji. The cell viability was assessed by the Alamar blue method. Result are mean $\pm$ SD of three different experiments. NHS: NHS alone; rILYd4: rILYd4 alone; RTX: RTX alone; OFA: OFA alone; IHS: heat inactive human serum; Triton X-100: total lysis; and rILY3: a nonfunction isotype of rILYd4.

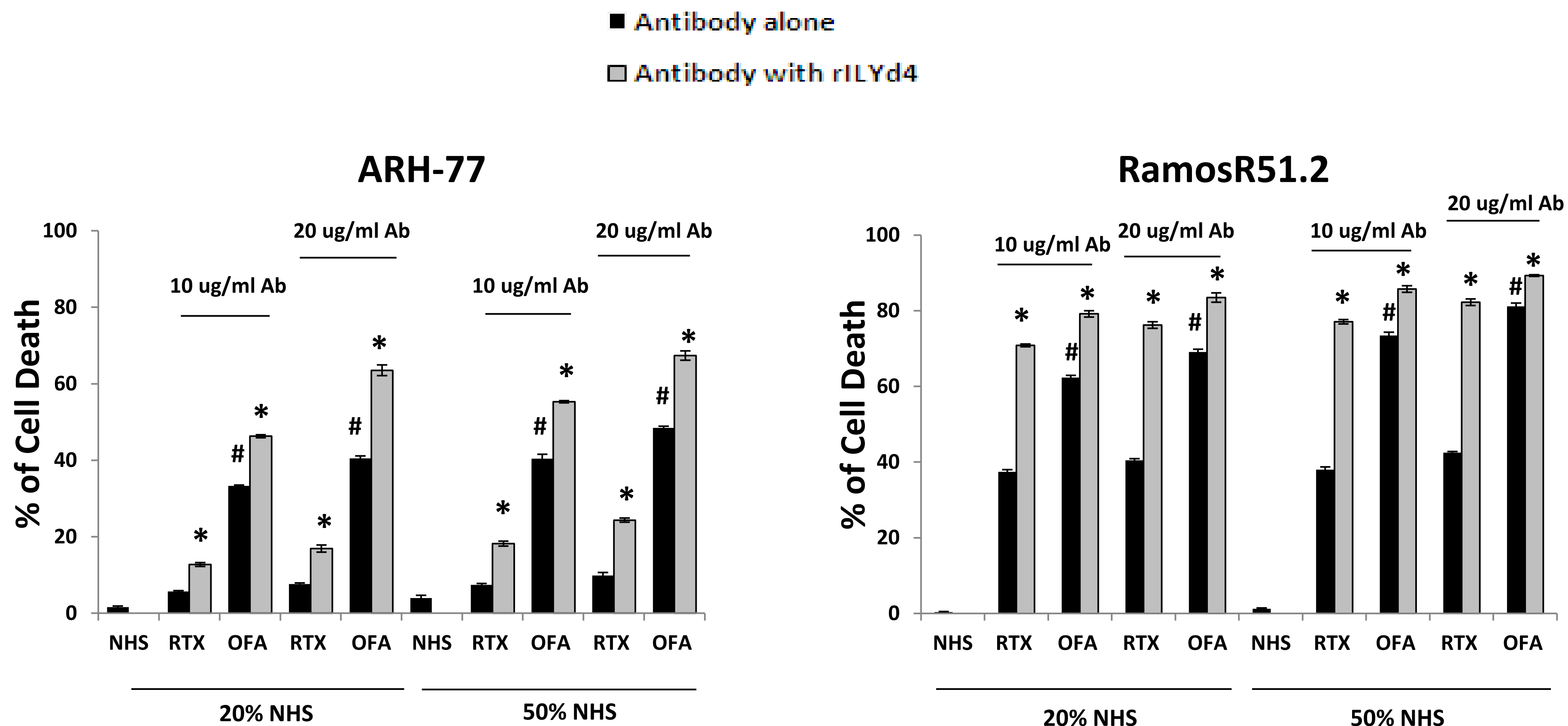
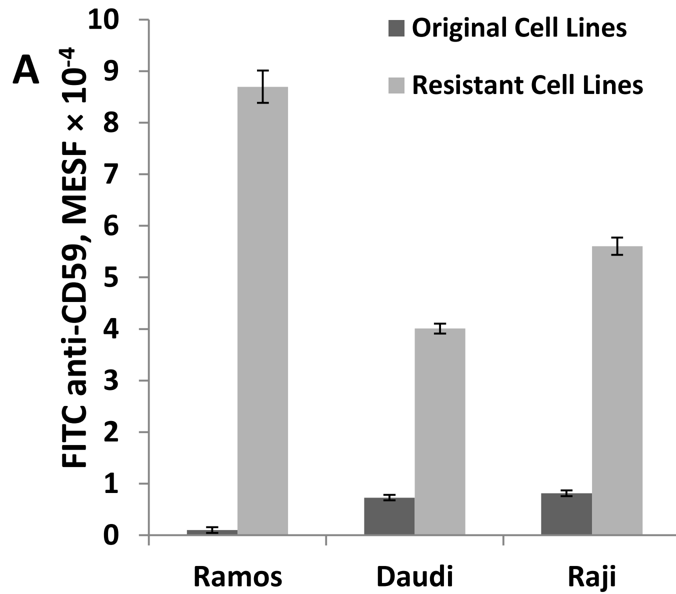


Figure S5: rILYd4 enhances RTX and OFA mediated CDC in B-cell malignancy cell line and RTX-resistant cell line *in vitro*. ARH-77 and RamosR51.2 were treated with different concentrations of RTX or OFA with 20% or 50% NHS in the absence or presence of 1074nM rILYd4. Cells were incubated at 37 °C for 2 hours. Cell viability was assessed by flow cytometry. Result are mean $\pm$ SD of three different experiments. Ab: Antibody. *: P < 0.01 v.s. no rILYd4 treatment, #: P < 0.01 v.s. RTX.



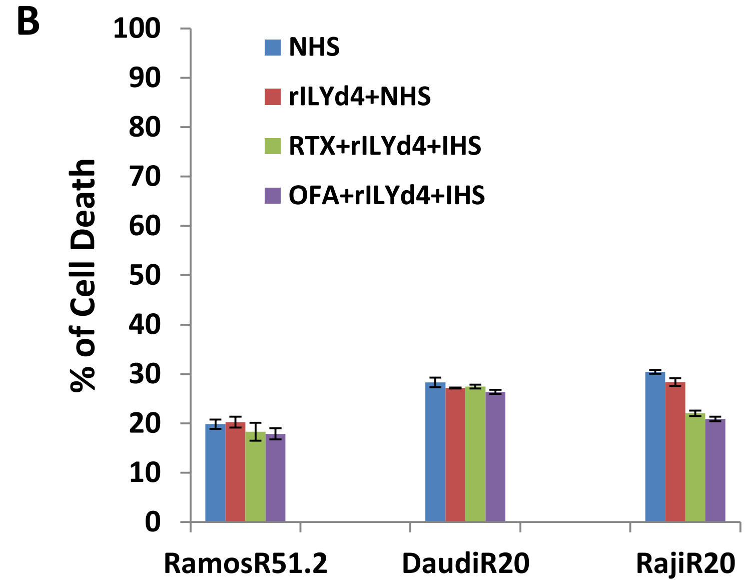
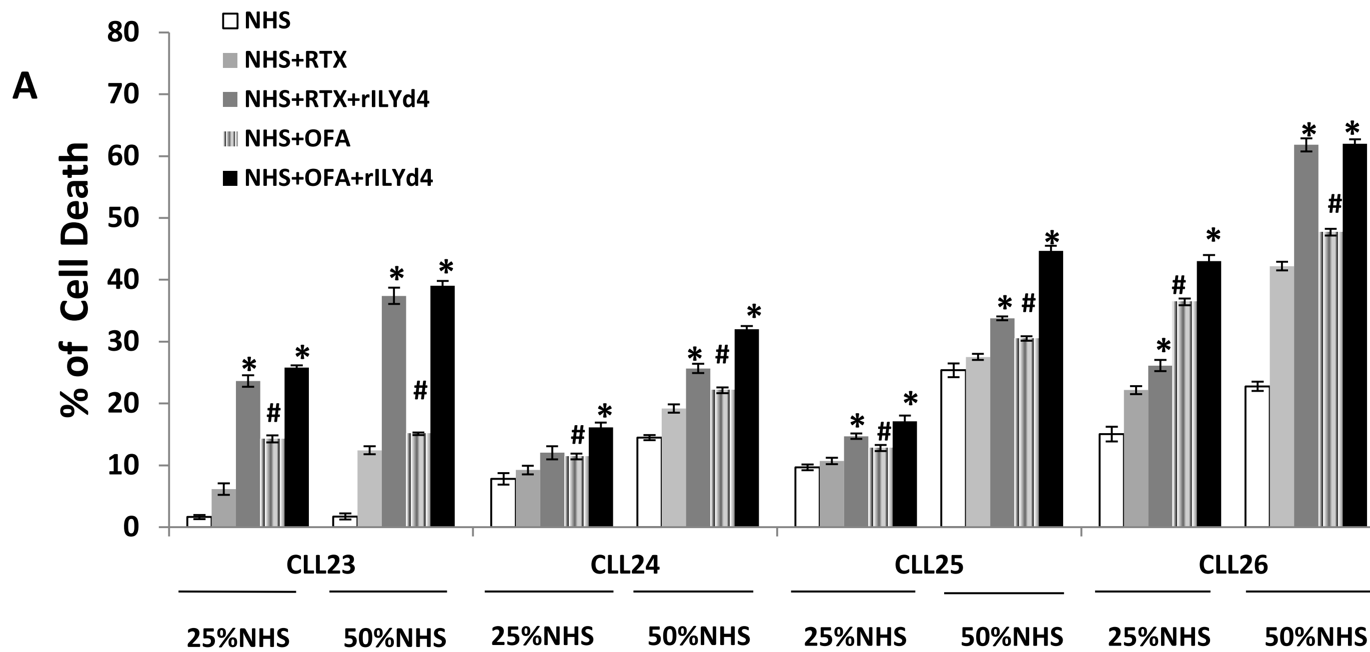


Figure S6: (A): CD59 expression level on original and RTX resistant B cell malignancy cell lines. Mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) using calibrated beads. Result are mean $\pm$ SD of three different experiments. **(B): Cell death after different treatments to Ramos R51.2, Daudi R20 and Raji R20 cells**. The concentration of rILYd4 is 1074 nM. The concentration of Abs is 20ug/ml. NHS: NHS alone.NHS/IHS is 20%. The cell viability was assessed by the PI staining method. Result are mean $\pm$ SD of three different experiments.



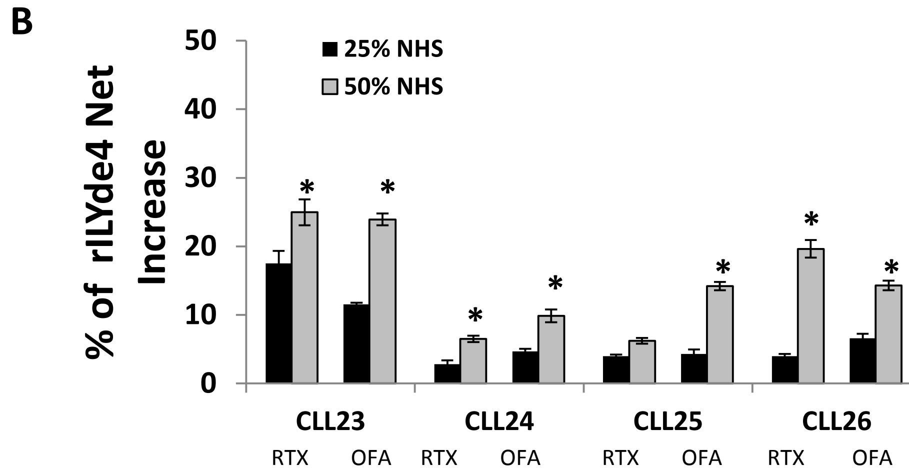


Figure S7: (A) rILYd4 enhances RTX and OFA mediated CDC on CLL cells *in vitro*. CLL cells were treated with 10 µg/ml RTX or OFA together with 25% or 50% NHS in the absence or presence of 1074nM rILYd4.Cells were incubated at 37 °C for 2 hours. Cell viability was assessed by flow cytometry. Result are mean ± SD of three different experiments. *: P < 0.01 v.s. no rILYd4 treatment, #: P < 0.01 v.s. RTX. **(B) The net percentage of rILYd4 increase of the CLL samples of 4 patients (CLL 23-CLL 26).** The data is presented as mean ± SD from the net percentage of cell death of mAb with rILYd4 subtracting the antibody alone induced cell death individually. *: P < 0.01 v.s. 25%NHS.

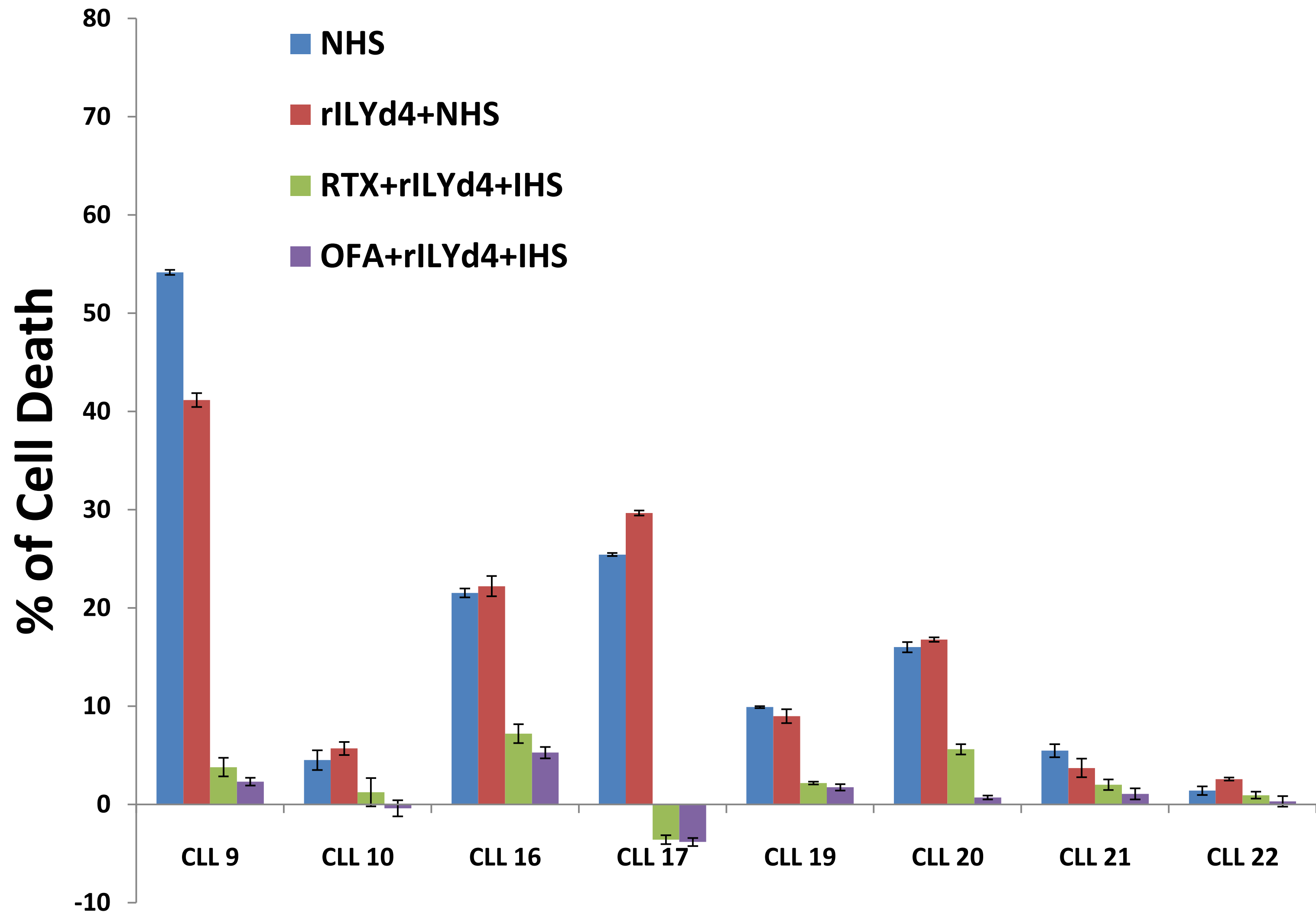
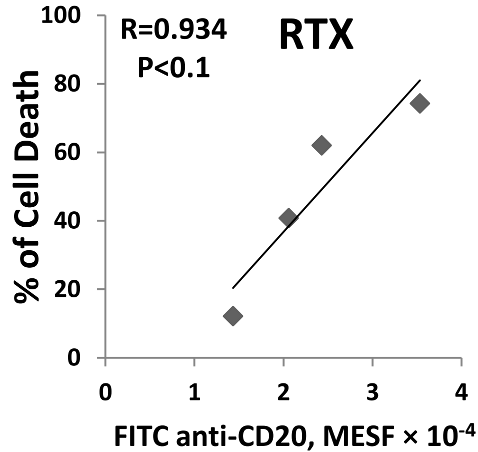


Figure S8: rILYd4 without RTX or OFA, or RTX or OFA with rILYd4 in the presence of inactivated HS does not increase cell death of 8 CLL samples. The concentration of rILYd4 was 1074 nM. The concentration of OFA or RTX was 10ug/ml. NHS: NHS alone. The NHS/IHS is 25%. Cell viability was assessed by the PI staining method. Result are mean ± SD of three different experiments.

A



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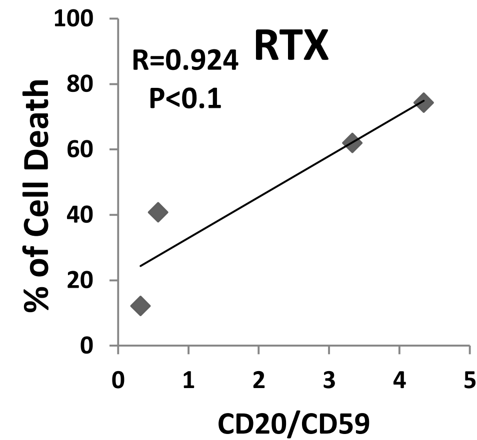

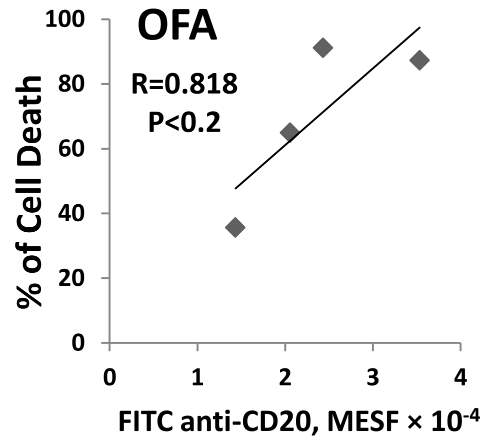

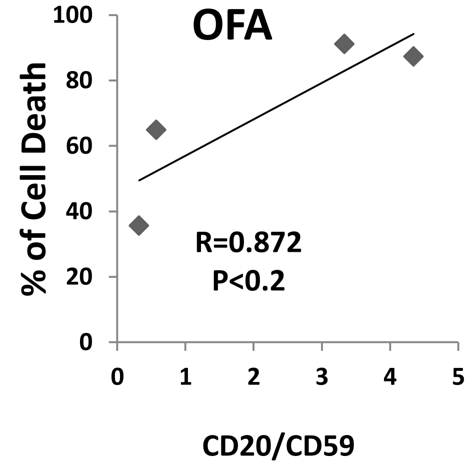

Figure S9: (A) CDC effects mediated by either OFA or RTX positively correlated with the level of CD20 on the cell lines ARH-77, RL, Daudi and Raji, though this effect did not reach significance. (B) **CDC effects mediated by either OFA or RTX positively correlated with the ratio of CD20/CD59 though this effect did not reach significance**.

Table S1: Clinical Characteristics of CLL patients

Patients	Date of DX	Date of Sample	WBC	B2M	% CD19+	% CD5+	%IgVH Homology	Mut/Unmut	ZAP +/-	FISH cytogenetics	Treatment
CLL 1	6/1/2001	1/15/2009	57.1	2.1	95.3	99.6	91.0	mut	Neg	24% 13q deletion	Untreated
CLL 2	6/6/2002	3/23/2010	36.1	2.8	95.1	97.6	96.0	mut	Neg	34% 13q deletion	Untreated
CLL 3	12/31/1993	11/23/2009	156.9	3.9	98.5	99.5	94.0	mut	Neg	72% 13q deletion	Untreated
CLL 4	1/1/1993	12/3/2009	69.6	2.3	94.0	98.0	94.7	mut	Neg	88% 13q deletion	Treated
CLL 5	12/31/2000	1/27/2010	95.9	5.4	95.0	95.0	100.0	unmut	Pos	67% trisomy 12 and 92% IgH rearrangement	Treated
CLL 6	8/16/2004	3/13/2006	48.6	2.2	89.1	96.8	98.6	unmut	Pos	83% 13q deletion	Untreated
CLL7	3/30/2009	8/24/2009	41.1	2.0	88.0	95.0	95.2	mut	Neg	70% 13q deletion and 88% IGH rearrangement	Untreated
CLL8	11/9/2001	4/28/2006	47.8	4.2	95.6	99.5	97.3	mut	Neg	78% 13q deletion, 5% 11q deletion, 10% 17p deletion, 9% IGH rearrangement	Untreated
CLL 9	1/1/1994	12/8/2005	243.0	3.4	96.3	99.0	95.4	mut	Neg	47% 13q deletion and 12% 17p deletion	Untreated
CLL 10	5/1/2008	2/13/2009	48.0	2.0	86.0	94.0	91	mut	Neg	81% 13q deletion and 3% 17p deletion	Untreated
CLL 11	7/28/2009	7/28/2009	21.2	1.8	75.0	92.0	Not Avail	Not Avail	Pos	58% 13q deletion and 4% 17p deletion	Untreated
CLL 12	6/1/2001	9/18/2007	15.5	3.5	Not Avail	Not Avail	97	mut	Pos	4/9/2002 showed 56% 13q deletion	Untreated
CLL 13	7/21/2003	3/20/2007	83.7	3.0	94.0	5.0	92.6	mut	Neg	3% trisomy 12, 5% 13q deletion, 3% 17p deletion	Untreated
CLL 14	2/15/2006	6/19/2009	43.0	1.6	Not Avail	Not Avail	91.7	mut	Pos	8% 13q deletion	Untreated

Table S1: Clinical Characteristics of CLL patients

Patients	Date of DX	Date of Sample	WBC	B2M	% CD19+	% CD5+	%IgVH Homology	Mut/Unmut	ZAP +/-	FISH cytogenetics	Treatment
CLL 15	2/1/2001	5/20/2008	27.4	1.4	72.1	90.0	98.6	unmut	Neg	66% deletion 13q	Untreated
CLL 16	6/1/2003	7/20/2007	92.1	3.1	96.0	73.0	87.6	mut	Neg	28% 13q deletion and 37% trisomy 12	Untreated
CLL 17	11/1/1998	8/16/2005	140.0	2.4	98.8	99.8	89.1	mut	Neg	90% 13q deletion	Untreated
CLL 18	12/12/2002	3/3/2006	55.1	2.8	73.4	94.1	92.2	mut	Neg	46% 13q deletion and 9% 17p deletion	Untreated
CLL 19	7/18/2000	4/28/2008	78	3.6	90.0	95.0	100	unmut	Pos	Trisomy 12	Treated
CLL 20	3/1/1989	3/16/2007	108	3.9	Not Avail	Not Avail	95.5	mut	Neg	24% 13q deletion	Treated
CLL 21	10/1/2002	2/15/07	58.6	4.0	91.0	93.0	100	unmut	Pos	91% 11q deletion and 83% 13q deletion	Treated
CLL 22	3/1/2004	3/19/2010	16.1	3.8	Not Avail	Not Avail	100	unmut	Neg	52% trisomy 12	Treated
CLL 23	3/22/2011	5/4/2011	283	Not Avail	Not Avail	Not Avail	96.5	mut	Neg	Not Avail	Untreated
CLL24	12/13/2010	3/3/2011	91.4	Not Avail	Not Avail	Not Avail	90.75	mut	Neg	52% mono del13q	Treated
CLL25	12/4/2008	11/22/2010	35.7	Not Avail	Not Avail	Not Avail	97.2	mut	Neg	63% mono del13q	Treated
CLL26	6/24/2010	9/16/2010	246.5	Not Avail	Not Avail	Not Avail	93.8	mut	Pos	98% mono 13q	Untreated

Note: DX: Diagnosis; WBC: white blood count, in K / μL; B2M: Beta-2-microglobulin, normal 0-2.7 mg/L; FISH: Fluorescence in situ hybridization; Neg: negative (<20% cells positive for ZAP-70); Pos: positive; Mut: mutated; Unmut: unmutated.