Materials

Cholesterol (Chol) and DSPE–PEG–maleimide (DSPE–PEG–Mal) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Methoxy-polyethylene glycol (MW=2000 Da)-distearoyl phosphatidylethanolamine (DSPE–PEG) and Egg phosphatidylcholine (egg PC) were obtained from Lipoid (Newark, NJ). 2-Iminothiolane (Traut's reagent), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of immunoliposome

Briefly, lipids Chol:Egg-PC:PEG-DSPE (molar ratio = 33.5: 65: 1.5) were dissolved in ethanol and a thin lipid layer was formed under rotavapor. Lipids were rehydrated in PBS and particle size was reduced by high pressure extrusion with nuclear polycarbonate membranes (pore sizes: 0.2 and 0.1 mm, Northern Lipids) as described previously.[1] Liposome size distribution was analyzed on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, CA). Volume weighted analysis showed particle size of approximate 100nm. A post-insertion method was adopted to incorporate antibody (milatuzumab) into preformed liposomes. In this method, milatuzumab was reacted with 10× Traut's reagent in Phosphate-buffered saline (PBS) (pH 8.0) for 2 hr at room temperature to yield sulfhydryl (SH) modified antibodies. Separation of milatuzumab-SH from unreacted 2- iminothiolane was performed using Sephadex PD-10 desalting column, and eluted with PBS (pH 6.5). The milatuzumab-SH was added to Mal-PEG-DSPE at a ratio of 10:1 (Mal-PEG-DSPE:milatuzumab-SH), and coupled overnight at room temperature. This antibody mixture was then incubated with liposomes for 1 h at 37^{o}C to form targeted ILP with antibody-to-lipid ratios of 1:1000. Non-coupled antibody was separated on a Sepharose CL-4B column using PBS (pH 7.4). All liposomal suspensions were filtered through 0.22 mM PES syringe filters to ensure sterility and milatuzumab-ILP was stored at 4^{o}C.

REFERENCE

1. Sapra P, Allen TM. Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. *Cancer Res.* Dec 15 2002;62(24):7190–7194.

Table S1. Patient characteristics

ID#	IgVH	del17p	Rai stage	Previous treatments	Flud resistant
CLL1	unmutated	no	0	Fludarabine (6 cycles, 2001), FCR (2004)	No
CLL2	mutated	no	III	no treatment to date	N/A
CLL3	mutated	yes	III	no treatment to date	N/A
CLL4	unmutated	yes	IV	Rituximab (2003, 2004, 2005, 2006)	N/A
CLL5	mutated	no	I	Fludarabine (2 cycles, 1998), Rituximab (2003)	No
CLL6	ND	no	I	no treatment to date	N/A
CLL7	mutated	no	I	no treatment to date	N/A
CLL8	unmutated	no	I	no treatment to date (However, started FCR on 2/11/08)	No
CLL9	mutated	no	II	no treatment to date	N/A
CLL10	ND	no	IV	no treatment to date	N/A
CLL11	unmutated	no	0	no treatment to date	N/A
CLL12	ND	no	I	Rituximab (2009)	N/A
CLL13	ND	no	I	no treatment to date	N/A
CLL14	unmutated	no	I	no treatment to date (for CLL)- started on CALGC 10404 shortly after (FR followed by lena)	N/A
CLL15	unmutated	no	III	no treatment to date	N/A
CLL16	unmutated	no	0	no treatment to date	N/A
CLL17	unmutated	no	III	no treatment to date	N/A
CLL18	ND	no	I	no treatment to date	N/A
CLL19	unmutated	no	IV	no treatment to date	N/A
CLL20	ND	no	I	Fludarabine & Ritux (2003)	No
CLL21	unmutated	no	III	Fludarabine (2002) Ritux (2003), CHOP (2009)	No
CLL22	mutated	no	0	no treatment to date	N/A
CLL23	mutated	no	I	Leukeran (2007)	N/A
CLL24	mutated	no	IV	Chlorambucil (1991-92, 1994, 1997), Fludarabine (2002), FR (05-06)	No
CLL25	unmutated	no	I	FR (2006-2007)	No
CLL26	unmutated	no	II	FR (2005), PCR (2007), PCR (2008) Bendamustine & Ritux (2009) Solu-medrol & Ritux (2010)	No
CLL27	mutated	no	0	no treatment to date	N/A
CLL28	unmutated	no	I	no treatment to date	N/A
CLL29	ND	no	I	no treatment to date	N/A
CLL30	unmutated	no	IV	no treatment to date (started FR on 3/29/10)	N/A

Figure S1. Milatuzumab induces apoptosis in CLL cells
MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assays were performed to determine a decrease in mitochondrial enzyme function (indicating cell death). One × 10^6 CD19-selected B-cells from CLL patients were incubated for 48 hours with anti-Fc crosslinking antibody, 5ug. μg/mL milatuzumab alone or the indicated dose of milatuzumab with 5 times excess anti-Fc crosslinking antibody for 48 hours. Fludarabine (1 uM) was used as a control to cell death. MTT reagent was then added, and plates were incubated for an additional 24 hours before spectrophotometric measurement.

Figure S2. CLL cells aggregate in culture following milatuzumab + anti-Fc treatment
Immunofluorescence staining (original magnification ×40) for CD74 in CLL patient cells either untreated, treated with 5 μg/mL milatuzumab alone or 5 μg/mL milatuzumab + 25 μg/mL anti-Fc crosslinking antibody for 48 hours. DAPI is used as a nuclear stain.

Figure S3. Soluble anti-Fc is required for milatuzumab induced CLL cell death
Viability by PI staining of CLL patient cells either untreated, treated with anti-Fc alone, 5 μg/mL milatuzumab, milatuzumab immobilized on a cell culture plate or milatuzumab + 25 μg/mL anti-Fc crosslinking antibody for 12, 24 and 48 hours. (N = 6; P <.0001 milatuzumab + anti-Fc vs. immobilized milatuzumab, averaged across all time points).

Figure S1 – Viability of CLL cells determined by MTT assay

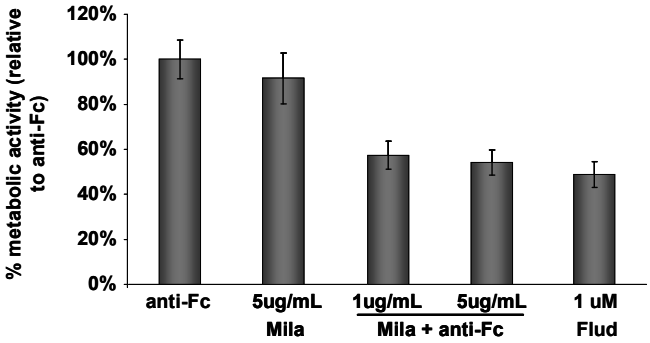

Figure S2 - CLL cells aggregate following milatuzumab + anti-Fc treatment

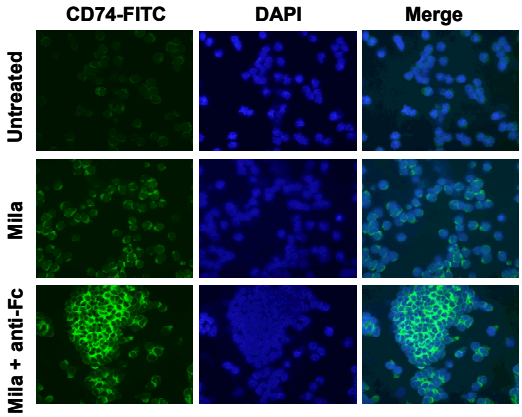

Figure S3 – Soluble anti-Fc is required for milatuzumab induced CLL cell death