Milatuzumab-Conjugated Liposomes as Targeted Dexamethasone Carrier for Therapeutic Delivery in CD74+ B-Cell Malignancies

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Corticosteroids are widely used for the treatment of B-cell malignancies, including non-Hodgkin’s lymphoma (NHL), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL); however, this class of drug is associated with undesirable off target effects. Herein we developed novel milatuzumab-conjugated liposomes as a targeted dexamethasone carrier for therapeutic delivery in CD74+ B-cell neoplasia and explored its effect against the disease.

These studies provide pre-clinical evidence and support for a potential use of CD74-targeted liposomal dexamethasone in overcoming dexamethasone-mediated adverse effects, and as a new therapy for B-cell malignancies.
Abstract

Purpose: Corticosteroids are widely used for the treatment of B-cell malignancies, including non-Hodgkin’s lymphoma (NHL), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL); however this class of drug is associated with undesirable off-target effects. Herein we developed novel milatuzumab-conjugated liposomes as a targeted dexamethasone carrier for therapeutic delivery in CD74+ B-cell malignancies, and explored its effect against the disease.

Experimental Design: The targeting efficiency of milatuzumab-targeted liposomes to CD74+ cells was evaluated in vitro. The effect of CD74-targeted liposomal dexamethasone was compared with free dexamethasone in primary CLL cells and cell lines in vitro. The therapeutic efficacy of CD74 targeted liposomal dexamethasone was evaluated in a Raji-severe combined immunodeficient (SCID) xenograft model in vivo.

Results: Milatuzumab-targeted liposomes promoted selective incorporation of carrier molecules into transformed CD74-positive B-cells as compared to CD74-negative T-cells. The CD74-dexamethasone-targeted liposomes (CD74-IL-DEX) promoted and increased killing in CD74-positive tumor cells and primary CLL cells. Furthermore, the targeted drug liposomes showed enhanced therapeutic efficacy against a CD74-positive B-cell model as compared to free, or non-targeted, liposomal dexamethasone (L-DEX) in SCID mice engrafted with Raji cells in vivo.
Conclusions: These studies provide evidence and support for a potential use of CD74-targeted liposomal dexamethasone as a new therapy for B-cell malignancies.
Introduction

Treatment options for patients with B-cell malignancies, including non-Hodgkin’s lymphoma (NHL), acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), are chemotherapy, antibody therapy, and corticosteroids. Potent corticosteroids such as dexamethasone (DEX) can induce apoptosis in malignant B cells by numerous pathways, including caspase activation, interleukin regulation, up-regulation of pro-apoptotic protein BIM and modulation of pro-survival factors, such as Bcl-2, Bcl-xL, AP-1 and NF-κB. Additionally, corticosteroids can antagonize microenvironmental stimuli that promote tumor cell survival and can act in a p53-independent manner. This is beneficial especially to patients who respond poorly to standard treatment due to p53 chromosomal abnormalities. While corticosteroids are highly active in virtually every type of B-cell malignancy, they have significant side effects, including immuno-suppression, hyperlipidemia, proximal muscle wasting, and osteoporosis. Developing a strategy to selectively target corticosteroids to B-cell malignancies represents an attractive treatment approach.

Liposomal delivery can alter drug pharmacokinetics, which can influence both efficacy and toxicity. Examples include clinically-approved liposomal daunorubicin (Daunoxome™) and liposomal doxorubicin (Doxil™) that have lower $C_{\text{max}}$ values and extended terminal half-lives as compared to their parental free drugs, and are associated with reduced cardiac toxicity. Liposomal corticosteroids have been evaluated for the treatment of arthritis and neoplasia in pre-clinical animal models. Immunoliposomes (ILs), i.e., liposomes coated...
with antibodies, can target delivery to cells expressing the specific antigen recognized by the antibody. Internalizing antibodies have been shown to be superior for therapeutic effects of ILs, but there has been limited evaluation of ILs for delivery of corticosteroids and to date, targeted delivery of corticosteroids to B-cell malignancies has not been explored. Development of ILs for corticosteroids would require a target antigen with relative B-cell selectivity and that mediates internalization.

CD74 has the attributes desired for ILs construction for B-cell specific targeting of corticosteroids. It is a type II transmembrane protein with increased expression on the surface of malignant NHL, ALL, and CLL cells and demonstrates robust internalization upon antibody binding. Milatuzumab, hLL1, is a humanized monoclonal antibody directed against CD74 that internalizes rapidly and is in clinical trials as a potential therapeutic for NHL, ALL, and CLL. We hypothesized that specificity of steroidal treatment could be enhanced using ILs coated with milatuzumab. We have shown previously that milatuzumab-ILs(CD74-ILs) potentiate the cytotoxic effect of the antibody, offering a potentially advantageous alternative to conventional treatments. Using this vehicle to transport DEX could represent a strategy for improving efficacy and reducing systemic toxicity. Herein, we present preclinical evaluation of CD74-IL-DEX for therapeutic efficacy in vitro and in vivo models of B-cell neoplasia.
Materials and Methods

Cell lines and primary CLL cells
Signed informed consent was obtained to procure cells from patients with previously diagnosed CLL as defined by the modified NCI criteria\textsuperscript{29}. Raji, Jurkat and 697 cell lines were obtained from the ATCC (Manassas, VA). Milatuzumab was provided by Immunomedics, Inc (Morris Plains, NJ). Trastuzumab (Genentech, San Francisco CA) and goat anti-human IgG antibody (Fc gamma fragment-specific, anti-Fc, Jackson ImmunoResearch Laboratories, West Grove, PA) were obtained commercially.

Flow cytometry assays
Viability was determined by staining with annexin V-FITC and propidium iodide (PI) as described previously\textsuperscript{30}. Milatuzumab was given at 5\(\mu\)g/ml and DEX at 10 \(\mu\)M. For surface staining, CLL cells were washed in PBS and stained with antibodies to CD19, CD20, CD74 (BD Biosciences, San Jose, CA) and/or fluorescence-labeled ILs. Cells were analyzed using a Beckman-Coulter model EPICS XL cytometer (Beckman-Coulter, Miami, FL). For internalization studies \(1\times10^6\) cells were treated with fluorescently-labeled antibodies for 30, 60, 120 minutes at 37\(^\circ\)C, washed with pH 3 glycine acidic buffer to remove surface bound antibodies, and processed for flow cytometry as previously described\textsuperscript{31}. Percent internalization at each time point was obtained by the formula (MFI[exp.] – MFI[0% control]) / (MFI[100%control] – MFI[0% control]) x 100. MFI: geometric mean fluorescence intensity; 100% control corresponds to CD74 expression on
the cell surface of cells treated with PBS alone; 0% control refers to cells incubated with isotype IgG as control at the designated time points.

**MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] Assay**

MTS assay was performed as previously described. Briefly, 1 × 10^6 cells were plated in 96-well plates and treated with appropriate drugs for 24 hours. Flavopiridol was used as a positive control. The plates were read 12 hours after addition of MTS solution and at 490 nm, and the absorbance values were normalized to media.

**Immunoblot analysis**

Immunoblots were performed as described. Antibodies used included GilZ (abcam, Cambridge, MA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

**Preparation of ILs**

CD74-ILs were prepared as previously described. Briefly, a post-insertion method was used to incorporate antibody into preformed liposomes, and CD74-ILs were prepared with antibody-to-lipid ratios of 1:1000. Methoxy-polyethylene glycol (MW=2,000Da)-distearoylphosphatidylethanolamine (PEG-DSPE) and egg phosphatidylcholine (Egg-PC) were obtained from Lipoid (Newark, NJ). Cholesterol (Chol) and DSPE–PEG–maleimide (DSPE–PEG–Mal) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). 2-Iminothiolane (Traut's reagent,) 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and other
chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). For liposome preparation, lipids [Chol: Egg-PC: PEG-DSPE (molar ratio = 33.5: 65: 1.5)] were dissolved in ethanol, dried to a thin film, and then re-hydrated with 0.2 M calcium acetate. The liposomes were dialyzed against HEPES-buffered saline (HBS, 145mMNaCl, 20mM HEPES pH7.4) overnight, using a DispoDialyzer (Spectrum Labs, Rancho Dominguez, CA) with a molecular weight cut-off of 10,000 Da. Liposome size distribution was analyzed by dynamic light scattering on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, CA). Volume weighted analysis showed an average particle size of 103 nm.

DEX-phosphate (Sigma-Aldrich, St. Louis, MO) was incubated with the liposomes at 37°C for 1 hour and then free drug was removed by Sepharose CL-4B column separation to yield liposomal DEX (L-DEX). A post-insertion method was adopted to incorporate antibody ligands into preformed L-DEX. In this method milatuzumab was reacted with 20 x Traut’s reagent (2 hours, room temperature) to yield sulfhydryl modified antibodies. The anti-CD74-SH was then reacted to micelles of Mal-PEG-DSPE at a molar ratio of 1:10, and then incubated with L-DEX for 1 hour at 37°C. ILs with CD74-PEG-DSPE-to-lipid ratios of 1:1000 were thus prepared.

Fluorescence-labeled liposomes and antibodies

Liposomes were fluorescently labeled with either calcein or octadecylrhodamine B chloride (R18) (Molecular Probes, Inc., Eugene, OR). Milatuzumab and the goat anti-human IgG antibody (Fc gamma fragment-specific, anti-Fc) were fluorescently conjugated with AlexaFluor 488 5-SDP ester (Invitrogen, Rockville,
MD), as described \(^{34}\). Localization of CD74-ILs and control IgG-ILs in Raji and Jurkat cells was examined by laser scanning confocal microscopy as described \(^{34}\).

**In vivo studies**

C.B-17 SCID (Taconic, Oxnard, CA) female mice were injected with 2 × 10^6 Raji Burkitt lymphoma cells through the tail vein using a mouse tail illuminator (Braintree Scientific Inc, Braintree, MA). DEX was given IP at 5mg/kg in free or liposomal form 3 times a week for 5 weeks. Antibodies were also given at 5mg/kg. Mice were monitored daily and sacrificed when they developed hind limb paralysis. Tissue samples obtained from tumor-bearing SCID mice that showed early signs of paralysis were submitted to Ohio State University Pathology Core Facility for histologic analysis to confirm the presence of human leukemic cells. In addition, bone marrow cells were obtained for flow cytometric analysis by flushing femurs with cold PBS following sacrifice. Cells were counted and stained with anti-human CD20 antibody and isotype controls for flow cytometry analysis. Absolute counts were obtained by multiplying total number of cells with percent CD20 positive cells. Animals were monitored daily for signs of illness and sacrificed immediately if hind-limb paralysis, respiratory distress, or over 20% body weight loss was noted. Survival time as determined by hind limb paralysis was the primary end point of the study.

**Statistical analysis**

All reported statistical evaluations were performed in the Center for Biostatistics at OSU. One way ANOVA was used to analyze cell line experiments. Linear
mixed-effects models were used for analyses of patient samples and in vivo experiments. Kaplan-Meier estimates of survival for treatments and engraftments were plotted, and the survival for each treatment was calculated with 95% confidence intervals. A significance level of $\alpha = 0.05$ was used for all tests. SAS software (version 9.2, SAS Institute, Inc., Cary, NC) was used for all statistical analyses.

Results

CD74-ILs are internalized in target CD74(+) tumor cells

Rapidly internalizing antibodies are preferred for ILs due to enhanced intracellular drug delivery and therapeutic effects$^{19,20,21}$. Previous work has established that the CD74 antibody, milatuzumab, can be internalized rapidly by target cells as compared to anti-CD19 and anti-CD20$^{27,36,37}$. We first evaluated CD74-ILs liposomes binding and internalization in vitro. Calcein-labeled CD74-ILs were tested in CD74(+) Raji Burkitt lymphoma cells$^{26,27,38}$ and CD74(-) Jurkat T lymphoblasts. Non-targeted liposomes were used as a negative control. We observed specific binding of CD74-ILs (representative shown in Figure 1A) to RajiB cells but not to control JurkatT cells (Raji MFI 14.7 vs Jurkat MFI 3.5, $P=0.002$). Control IgG-ILs (MFI 3.9, Figure 1A) and non-conjugated liposomes (MFI 2.8, Figure 1B) did not bind specifically to Raji cells. Collectively, this demonstrated specificity of CD74-ILs for the CD74(+) target cells.

Next we sought to determine the efficiency of CD74-ILs internalization into Raji cells. In Figure 1C we demonstrated that CD74-ILs can be
internalized rapidly into target cells similar to that observed with anti-CD74 antibody alone (n=3, CD74-ILs vs. CD74 P>0.20 for 30, 60, 120 minutes, respectively). In contrast, IgG-ILs showed no internalization with similar MFI throughout the time points, which was thus used for normalization of results. To confirm these findings and determine the localization of the CD74-ILs in the target cells, we performed confocal microscopy. Our findings further demonstrated that CD74-ILs were localized to the cell membrane and were internalized in the target Rajicells (Figure 2 panels A, B, and G), whereas controls did not. CD74-ILs (panel I) did not bind nor internalize in CD74(-) Jurkat cells. Collectively, these results indicate that CD74-ILs can bind with specificity to target cells and are internalized rapidly, which justifies further development of CD74-ILs.

Creation of CD74-ILs containing DEX

CD74-ILs loaded with DEX were synthesized. Milatuzumab anti-CD74 antibody was incorporated after drug loading. The ILs had a mean size of 103 +/- 12nm. The drug was incorporated by remote loading with a pH gradient generated by calcium acetate \(^{33,39}\). The efficiency of drug loading of the particle was 92-94% (data not shown).

In vitro activity of CD74-IL-DEX

CD74-IL-DEX was tested for in vitro cytotoxicity against lymphoid cell line and primary CLL cells. Previously we have shown that in vitro CD74-ILs are highly effective in killing B-CLL cells and mimic cross-linked CD74 mediated cytotoxicity\(^ {28}\). Primary CLL B cells were incubated for 24 hours with CD74-ILs,
CD74 with cross-linker, CD74-IL-DEX, or free DEX. The cells were stained with propidium iodide (PI) and processed for flow cytometry. The results shown in Figure 3A indicate that CD74-IL-DEX can induce apoptosis to CLL B cells to a higher degree than empty CD74-ILs (n=14, % PI positive cells 25.07 vs. 15.92 respectively, \( P<0.0001 \)) and free DEX (PI positive cells 16.03, \( P<0.0001 \)). Cross-linked milatuzumab had comparable levels of PI positive staining as CD74-IL-DEX (27.92 vs. 25.07, respectively). Similar results were observed examining viability by assessment of mitochondrial activity, as shown in Figure 3B. In Figure 3A we observed that L-DEX-treated cells had lower PI positive staining than free DEX (10.41% vs. 16.03% PI+, respectively, n=14, \( P=0.003 \)) and higher MTS assessed mitochondrial activity than free DEX (Figure 3B, n=8, 77.62% vs. 46%, \( P<0.0001 \), respectively). This implies that the encapsulation of DEX into liposomes altered the free drug dynamics over time and diminished the efficacy. In our previous study with Raji cells, milatuzumab-ILPs showed potent apoptosis due to liposome mediated cross-linking of the milatuzumab used\(^{28}\). In order to appreciate the pharmacological effect of DEX in CD74-ILs encapsulated DEX formulation, we optimized the concentrations of milatuzumab in CD74-IL-DEX formulation to a suboptimal level so that the CD74-ILs effects were minimized and the effects of the delivered DEX could be evaluated. Our findings indicate that encapsulating DEX into liposomes and targeting it with milatuzumab can induce target cell apoptosis and affect normal cellular functions, resulting in significant apoptosis of target cells compared to either milatuzumab or empty...
CD74-ILs at the corresponding concentration, as shown with Raji lymphoma (Figure 3C) and 697 acute lymphoblastic leukemia (Figure 3D) cell lines. To further confirm the cytotoxicity of CD74-IL-DEX is dependent upon DEX rather than CD74-ILs, immunoblot analysis on the glucocorticoid induced leucine zipper (GilZ) protein was selected to show cellular GR level response (Figure 4). This demonstrates that after 48 hours treatment with DEX at 10μM and CD74 accordingly, the levels of GilZ were induced by CD74-IL-DEX and L-DEX, but not significantly by other treatment, including free DEX.

**CD74-IL-DEX increases survival of SCID mice bearing Raji xenografts.**

The *in vitro* cytotoxicity of CD74-IL-DEX showed promising results that justify further *in vivo* evaluation. Therefore, we sought to determine the effectiveness of the CD74-IL-DEX as a therapeutic agent. The disseminated Raji xenograft model described by our group previously was obtained by engrafting SCID mice with Raji cells. The malignant cells infiltrated the central nervous system and bone marrow and, within approximately two weeks, mice developed hind leg paralysis requiring sacrifice. This disease model is commonly used to study lymphoproliferative malignancies and is pertinent for our *in vivo* studies, since CD74-ILs specificity has been shown with Raji cells. Three days post engraftment mice were treated with CD74-IL-DEX (n=16), empty CD74-ILs (n=16), CD74 (n=16), free DEX (n=8), or L-DEX (n=8). Control groups also included Herceptin-IL-DEX (n=16), Herceptin-ILs(n=8), Herceptin (n=7), empty liposomes (n=7), and PBS (n=8). Treatment was administered with intraperitoneal injections three times a week for five weeks. Antibodies and drug
were given at 5mg/kg in free or liposomal form. All control mice died within two weeks post engraftment due to hind limb paralysis. As seen in Figure 5, milatuzumab anti-CD74 antibody alone increased survival over control groups, but mice treated with CD74-ILs survived approximately 6.35 days longer than CD74 alone ($P=0.04$) corroborating our previous *in vitro* data that CD74-ILs enhanced direct killing of this antibody mentioned earlier and also shown previously. Mice receiving CD74-IL-DEX survived longer than all other groups ($n=16$, $P=0.0002$). The CD74-IL-DEX group survived an average of 13.56 days longer (Table 1) than the CD74-ILs group, and 29.4 days longer than control groups. Consistent with this finding, flowcytometric analysis of bone marrow from CD74-IL-DEX treated mice showed reduced human CD20+ cells (25%, 38% and 40% hCD20+ cells in CD74-IL-DEX, CD74-ILs and CD74 treated groups respectively). These results suggest that incorporating DEX into liposomes and delivering it with anti-CD74 to malignant cells can achieve better survival rates than the drug or antibody given separately.

**Discussion**

Treatment schemes for B-cell malignancies such as CLL, NHL and ALL commonly include corticosteroids, such as dexamethasone. This potent drug has great immunosuppressive, anti-inflammatory and pro-apoptotic properties desirable for malignancies, as well as other diseases like asthma and arthritis. Nonetheless, the benefits of corticosteroids sometimes are compromised by their numerous side effects. Here, we describe how encapsulation of DEX into
liposomes and delivery with milatuzumab, anti-CD74 antibody can achieve target specificity and improved therapeutic effects. Our findings show that CD74-ILs bind and are internalized in CD74(+) Raji cells, offering a robust vehicle for drug encapsulation. CD74-IL-DEX induced cell death to malignant primary CLL cells and disturbed mitochondrial activity to a higher degree than cross-linked antibody or empty CD74-ILs. Free DEX and L-DEX did not mediate such effects, demonstrating the need for drug encapsulation and antibody to improve selective targeted delivery. The promising \textit{in vitro} results obtained were further evaluated in SCID mice engrafted with CD74+ Rajicells. These studies demonstrated that CD74-IL-DEX treatment significantly prolonged survival of mice by 29.4 days as compared to control groups ($P=0.0002$). This was also consistent with the decreased human CD20+ cells observed in the bone-marrow of the CD74-IL-DEX treated group. The bone marrow microenvironment provides survival and growth advantage for leukemic B cells \cite{44} and reduction of malignant B cells in the bone marrow using CD74-IL-DEX has clinical relevance to the disease. Consistent with this, treatment with CD74-IL-DEX exhibited superior \textit{in vivo} efficacy compared to non-targeted and control formulations.

In the current therapy studies we used 5 mg/kg of antibodies and DEX, based on previous data and our preliminary studies \cite{27,34}. We also adopted the 5 week treatment scheme to mimic clinical dosing of milatuzumab \cite{45,46} and to address the aggressiveness of the Raji xenograft model. Effects of various dosing schemes of CD74-IL-DEX and alterations in the drug and antibody ratios that can alter the therapeutic \textit{in vivo} efficacy of the used formulations remains to be evaluated. Since
preliminary invitro studies failed to show significant advantage in combining CD74-ILs with free DEX, we focused our invivo analysis to evaluate the efficacy of targeted delivery using CD74-IL-DEX and compared to isotype control Herceptin-IL-DEX and free DEX.

Numerous studies have established that liposomal drugs behave differently than their free counterpart\textsuperscript{15,47,48}. Here we also demonstrate that L-DEX has different effects than free drug \textit{in vivo}. Results from a pilot pharmacokinetic study indicated that free and L-DEX had different pharmacokinetics (manuscript in submission). Our findings showed that the \( C_{\text{max}} \) of L-DEX was approximately two times lower than free DEX. The area under the curve was about 1.4 times higher with L-DEX versus free DEX. The clearance of free drug was also faster than L-DEX and the bioavailability of the L-DEX was higher than free DEX, likely due to slower drug clearance as compared to free drug. Furthermore, the study presented in ICR mice showed that L-DEX did not alter lymphocyte numbers from the spleen, but free drug reduced CD19(+) splenic lymphocytes, supporting our \textit{in vitro} cytotoxicity data (data not shown). Interestingly, this effect was not seen with CD3(+) lymphocytes, which remained similar in both free and L-DEX groups.

There are multiple studies showing that corticosteroids induce lymphocytic apoptosis, but the way that DEX induces apoptosis in lymphocytes is intricate, variable and has not been fully elucidated\textsuperscript{40,41,42}. For mature T cells, studies in peripheral lymphocytes have shown that the effect is mainly attributed to redistribution of cells to other compartments. The effect could be specific to spleen cells and dependent on the dosing and endpoint of the study, and could
indicate a higher sensitivity of B cells in spleen to DEX apoptosis as compared to T cells. Regardless, the findings are intriguing and additional studies could possibly explain this observation. In the same study we also observed that free DEX increased serum cholesterol levels significantly post treatment (supplementary Figure S1), a common corticosteroid side effect. Interestingly, in contrast to free DEX, LP-DEX failed to alter serum cholesterol levels. The aggressive Raji-SCID model precluded cholesterol evaluation beyond 2 week time. Nevertheless, the CD74-IL-DEX induced less cholesterol levels compared to free DEX. Development of appropriate leukemic mouse models expressing human CD74 will allow better characterization of toxicity associated with targeted formulations in longer duration. The results show that liposomal drug has a different action than free drug and the potential to avoid free drug-associated side effects. Thus, our results indicate that L-DEX has preferred attributes over the free drug, which are further enhanced by targeting with anti-CD74. The lack of clinical advantage with corticosteroid liposomes is likely to be overcome with targeted delivery formulations. Detailed pharmacokinetic profile of immunoliposomal drug will allow defining the clinical advantage of this formulation. Nevertheless, previous and ongoing studies indicate faster plasma clearance of IL-formulated drugs presumably through efficient delivery and capture by the targeted cells or tissues. In this context, it is possible to increase the dose of DEX delivered to desired cell type minimizing adverse toxicity.

Limited studies have been performed with corticosteroids carried in antibody directed ILs. Most corticosteroid liposomes have been tested in non-
hematologic malignancy models and do not offer the advantage of antibody targeting. Here, we present an innovative strategy to package DEX into CD74-ILs and thereby alter the properties of the drug, limit side effects and enhance its efficacy. IL delivery for hematologic malignancies is likely to have added benefit as opposed to solid tumors, due to the fact that many of the malignant cells are readily accessible in the blood stream. Moreover, keeping liposome size to 100-200 nm range can achieve better specificity into tumor homing compartments such as the bone marrow and achieve extended drug circulation time \(^{49,50}\).

The findings demonstrate therapeutic enhancement of DEX targeted with CD74-ILs. Our studies can have applications to various B-cell malignancies treated with corticosteroids. Furthermore, CD74-IL-DEX could serve as platform for other corticosteroid liposome development with different antibodies to achieve disease-specific therapy. The post insertion method we have used, however, warrants conjugation of milatuzumab onto the surface of liposome, and this may result in random orientation of the conjugated antibodies. We are currently working on combining an anti-CD20 antibody with milatuzumab for dual targeting of ILs to target cells. We speculate that the combination of rapidly internalizing milatuzumab and clinically established rituximab or other anti-CD20 antibodies may offer an added advantage to L-DEX targeting. Overall, this theme seeks to pursue novel ways to administer drugs that are active but limited by their toxicity. Our work is an example of how we can successfully develop ILs for hematologic malignancies and combine antibodies with drugs in one system for ease of use,
enhanced targeting, and improved pharmacokinetics with the goal of improving treatment of these diseases.

**Author Contributions:** YM designed and performed experiments, contributed to draft and revisions of the paper, completed the paper and approved the final submitted version. GT designed and performed experiments, wrote the first draft of the manuscript, contributed to revisions of the paper, and approved the final submitted version. EH, WT, MS, provided input into experimental design, performed experiments, and reviewed and approved the final version of the manuscript. MX and DJ assisted in design of experiments, performed the statistical analysis reported, reviewed and approved the final version of the manuscript, MP, LYL and GM provided input into experimental design, reviewed drafts of the manuscript and approved the final submitted version. DMG provided essential materials, revised the manuscript, and approved the final version. RJL, NM, and JCB obtained funding to perform the research, designed the experiments, participated in the analysis of the data, review of multiple drafts of the manuscript and approved the final version for submission.

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References


Figure legends

**Figure 1. CD74-ILs bind and are internalized into CD74(+)Raji cells.**

CD74-ILs labeled with calcein is shown by flow cytometry to bind CD74(+)Raji B cells (A), but not CD74(-)Jurkat T cells (B). Non-specific IgG-ILs do not bind Raji cells (A,B). Internalization of CD74-ILs and anti-CD74 (n=3) is shown in Raji cells (C), over time portrayed by change in Mean Fluorescent Intensity (MFI). The results were normalized to IgG isotype and IgG-ILs.

**Figure 2. Localization of CD74-ILs in target cells**

CD74-ILs, after 1 hour incubation with Raji cells (panel A,B,G,J) visualized by confocal microscopy. CD74-ILs are observed inside the cells and also on the cell membrane. Controls such as IgG-ILs (panels C,L) and non-targeted liposomes (panels D,E,K,) did not enter/bind the cells. CD74-IL in CD74(-) cell line Jurkat (panel I) did not bind/enter the cells. All lipids are R18-labeled.

**Figure 3. CD74-IL-DEX decreases viability and mitochondrial activity in CLL cells**

Primary CLL cells (n=14) were treated for 24 hours with DEX (10 μM) in free or liposomal forms with cross-linked milatuzumab (CD74+Fc) and all CD74 concentrations at 5 μg/ml (A). Flow cytometry analysis of percent propidium iodide (PI) positive cells indicated that CD74-IL-DEX kill significantly more CLL B cells than CD74-ILs (P-value<0.0001) and DEX (P-value<0.0001). MTS assay analysis (B) shows mitochondrial activity in CLL primary cells (n=8) after 24 hours of treatments with similar conditions as for flow assay. CD74-IL-DEX reduces mitochondrial activity at a higher degree than CD74-IL (P-value<0.0001) and L-DEX (P-value<0.0001). CD74-ILs are significantly different from CD74+fc.
(P-value<0.0001) for both assays. Figure 3C shows 24, 48 hours Annexin V, PI staining in Rajicells (n=3). Figure 3D shows 24, 48 hours Annexin V, PI staining in 697 cells (n=3). Free DEX with CD74 or CD74-ILs does not significantly alter cytotoxicity of cells in comparison to free CD74 antibody or CD74-ILs, however, liposomal DEX kills cell significantly compared to free DEX and CD74.

**Figure 4.** CD74-IL-DEX and L-DEX increase cellular GilZ level. Immunoblot analysis of GilZ induction in whole cell lysates isolated from Raji cells treated with different formulations of DEX at 10μM and equivalent CD74.

**Figure 5.** CD74-IL-DEX extends survival of SCID mice bearing Raji xenografts. Shown on top, survival curve of SCID mice treated with various drugs. All control groups died within days 14-15 days post engraftment. CD74-ILs increased the mean survival by 6.35 days compared with CD74 treatment alone (P-value=0.0405). CD74-IL-DEX Liposome group survived an average of 13.56 days longer than CD74-ILs group (P-value=0.0002) and increased mean survival by 29.4 days in the compared with controls (P-value=0.0002). Figure on bottom shows statistical analysis using ANOVA to compare mean days of survival among groups. A summary of statistical results is presented in Table 1.
Figure 1
Figure 2
Figure 3A
Figure 3B

% Mitochondria Activity Relative to Media Control

- DEX
- Liposome
- L-DEX
- CD74-ILs
- CD74-IL-DEX
- CD74+Fc
- Flavopiridol

- * Patient
- - Mean
Figure 3C
Figure 3D
Figure 4

Untreated

Free DEX

CD74+Fc

CD74-ILs + free DEX

CD74-ILs

CD74-IL-DEX

L-DEX

GAPDH

β-actin
Figure 5, top
### Table 1. Survival statistics from SCID Raji xenograft study

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Estimate (Difference in days of survival)</th>
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<th>*95% CI</th>
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<td>A: CD74-ILs vs CD74</td>
<td>6.19</td>
<td>2.77</td>
<td>9.61</td>
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<tr>
<td>B: HERCEPTIN-ILs vs HERCEPTIN</td>
<td>-0.16</td>
<td>-5.17</td>
<td>4.85</td>
</tr>
<tr>
<td>A-B: Interaction between Liposome and CD74</td>
<td>6.35</td>
<td>0.0405</td>
<td>0.28 12.41</td>
</tr>
<tr>
<td>C: CD74-IL-DEX vs CD74-ILs</td>
<td>13.56</td>
<td>10.41</td>
<td>16.98</td>
</tr>
<tr>
<td>D: HERCEPTIN-IL-DEX vs HERCEPTIN-ILs</td>
<td>0.13</td>
<td>-4.07</td>
<td>4.30</td>
</tr>
<tr>
<td>C-D: Interaction between CD74 and DEX (with liposome)</td>
<td>13.44</td>
<td>0.0002</td>
<td>8.03 18.84</td>
</tr>
<tr>
<td>CD74-IL-DEX vs HERCEPTIN-IL-DEX</td>
<td>29.4</td>
<td>0.0002</td>
<td>25.95 32.78</td>
</tr>
</tbody>
</table>
Clinical Cancer Research

Milatuzumab-Conjugated Liposomes as Targeted Dexamethasone Carrier for Therapeutic Delivery in CD74+ B-Cell Malignancies

Yicheng Mao, Georgia Triantafillou, Erin Hertlein, et al.

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