Combination anti-CD74 (milatuzumab) and anti-CD20 (rituximab) monoclonal antibody therapy has in vitro and in vivo activity in mantle cell lymphoma

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Running title: Preclinical evaluation of milatuzumab and rituximab in mantle cell lymphoma

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Abstract

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy with a median survival of 3 years despite chemo-immunotherapy. Rituximab, a chimeric anti-CD20 monoclonal antibody (mAb), has shown only modest activity as single agent in MCL. The humanized mAb milatuzumab targets CD74, an integral membrane protein linked with promotion of B-cell growth and survival, and has shown preclinical activity against B-cell malignancies. Since rituximab and milatuzumab target distinct antigens and potentially signal through different pathways, we explored a preclinical combination strategy in MCL. Treatment of MCL cell lines and primary tumor cells with immobilized milatuzumab and rituximab resulted in rapid cell death, radical oxygen species generation, and loss of mitochondrial membrane potential. Cytoskeletal disrupting agents significantly reduced formation of CD20/CD74 aggregates, cell adhesion and cell death, highlighting the importance of actin microfilaments in rituximab/milatuzumab-mediated cell death. Cell death was independent of caspase activation, Bcl-2 family proteins or modulation of autophagy. Maximal inhibition of p65 nuclear translocation was observed with combination treatment, indicating disruption of the NF-κB pathway. Significant in vivo therapeutic activity of combination rituximab and milatuzumab was demonstrated in a preclinical model of MCL. This data supports clinical evaluation of combination milatuzumab and rituximab in MCL.
Introduction

Mantle cell lymphoma (MCL) is a B-cell malignancy with a variable histology and clinical course, distinguished by the characteristic translocation t(11;14)(q13,q32) which results in over-expression of cyclin D1 and consequent dysregulation of cell cycle control.1 Additionally, MCL exhibits alterations in cell survival pathways including constitutive activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling2 and nuclear factor-κB (NF-κB).3 Despite the hallmark genetic translocation in MCL, the clinical course of MCL is variable with some patients experiencing indolent disease4 while others exhibit rapid progression.5 MCL patients have a median overall survival (OS) of approximately three years, and no consensus exists for standard first-line therapy.6-9 While aggressive therapies including chemo-immunotherapy10,11 or stem cell transplant12,13 have been shown to improve outcomes, no therapy offers the potential for cure. Given the absence of curative therapy and the limited number of options for patients with relapsed/refractory MCL, novel treatment approaches are essential.

Rituximab (Genentech Inc, San Francisco, CA), a chimeric anti-human CD20 monoclonal antibody (mAb), has been utilized in multiple strategies to treat patients with MCL.14 As a single agent, rituximab has been tested in patients with newly diagnosed and relapsed/refractory MCL with response rates (RR) of 27 to 38% and a median response duration of 6 to 12 months.15,16 Interestingly, the RR obtained in untreated patients was not higher than in relapsed/refractory patients, relegating this antibody to the group of modestly active agents in MCL. However, in combination with anthracycline-based regimens, RR and time to progression, but not OS, of treatment-naive MCL patients was significantly increased when compared to patients treated with chemotherapy alone.17
Milatuzumab (hLL1, IMMU-115, Immunomedics Inc., Morris Plains, NJ) is a fully humanized IgG1κ mAb specific for CD74, a type II transmembrane glycoprotein associated with MHC class II α and β chains. CD74 was originally thought to function as an MHC class II chaperone; however, was recently found to also play an important role as an accessory signaling molecule and survival receptor in the maturation and proliferation of B-cells by activating the PI3K/Akt and the NF-κB pathways.18-20 CD74, which is quickly internalized upon binding with its physiologic ligand, the macrophage migration-inhibitory factor (MIF),21 is expressed on the majority of B-cell malignancies, making it an attractive therapeutic target. CD74 is also expressed on normal B-cells, monocytes, macrophages and dendritic cells (DC).22 However, it has been recently shown that milatuzumab has minimal effects on the viability of normal B-cells and DCs.23 Furthermore, it has been shown that milatuzumab has no effect on DC maturation and DC-mediated T-cells function.23 Milatuzumab demonstrated anti-proliferative activity in transformed B-cell lines, improved survival in preclinical models,18,22 and is presently being evaluated in clinical trials for the treatment of several hematologic malignancies (NCT00421525; NCT00868478; NCT00603668; NCT00504972). Unlike rituximab, milatuzumab does not cause cell death via antibody dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).22,24

Rituximab and milatuzumab target distinct antigens lacking known association and, as single agents, have demonstrated substantial anti-tumor activity in B cell non-Hodgkin’s lymphoma (NHL) cells,22,25 providing the rationale for exploring this combination treatment strategy in MCL. From a translational standpoint, dual antibody therapy offers several advantages including: favorable toxicity profiles which may permit frequent
dosing or maintenance treatment; additional treatment options for heavily pre-treated patients or patients with significant comorbidities; potentially increased efficacy when compared to single agent regimens due to alternative mechanisms of action; and the ability to overcome resistance mechanisms that may evolve in the setting of single agent mAb therapy. Herein, we report the preclinical in vitro and in vivo activity of combination milatuzumab and rituximab and explore the mechanism by which this combination treatment induces cell death in MCL.

Materials and Methods

Primary tumor cells and cell lines

Primary tumor cells were isolated from the peripheral blood of patients with MCL after obtaining informed consent in accordance with the Declaration of Helsinki detailed in a protocol approved by the Ohio State University Institutional Review Board. All patients studied (clinical characteristics summarized in Figure 2B) were diagnosed with MCL according to the World Health Organization (WHO) classification of tumors.26 Characteristics of the MCL cell lines, summarized in Table 1, have been previously described. Jeko, Mino and SP-53 were generously contributed by Dr. Raymond Lai, University of Alberta, Edmonton, Alberta, Canada. Rec-1, Granta-519, and HBL-2 were generously contributed by Dr. Owen A. O’Connor.27

Reagents

Rituximab (Genentech Inc, San Francisco, CA) and trastuzumab (Genentech Inc, San Francisco, CA) were obtained commercially. Milatuzumab and veltuzumab were provided by Immunomedics (Immunomedics Inc, Morris Plains, NJ). Ofatumumab was
supplied by Genmab (Genmab Inc, Princeton, NJ). Q-VD-OPH pan-caspase inhibitor was purchased from MP Biomedicals (Solon, OH).

**Immunophenotypic studies**
Immunophenotyping was performed to determine the percentage and mean fluorescence intensity (MFI) of CD20 and CD74 on MCL cell lines and primary tumor cells relative to a specific isotype control. Further details are available in supplemental materials.

**Synthesis of fluorescence-labeled antibodies**
Rituximab and the goat anti-human IgG antibody (Fc gamma fragment-specific, anti-Fc) (Jackson ImmunoResearch Laboratories, West Grove, PA) were fluorescently conjugated with N-Hydroxysuccinimide (NHS)-ester rhodamine (Pierce, Biotechnology, Rockford, IL). Milatuzumab and total p65 were fluorescently conjugated with alexa fluor 488 5-SDP ester (Invitrogen, Rockville, MD), as described. Further details are available in supplemental materials.

**Assessment of antibody-binding and antigen surface density**
Quantitative analysis of CD74 and CD20 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, IN) according to manufacturer’s instructions. Further details are available in supplemental materials.

**Analysis of cell death by flow cytometry**
Cell viability was measured by dual staining with Annexin-V FITC and Propidium Iodide (PI) (BD Pharmingen, San Diego, CA) as described. ROS generation, mitochondrial
membrane potential ($\Delta \psi_m$) changes studies and actin reorganization experiments were performed as described. Further details are available in supplemental materials.

**Immunoblot analysis**

Immunoblots were performed as described. Antibodies to the following proteins were used: caspase 3, 2, PARP and total p65/NFκB (Cell Signaling Technology, Danvers, MA); actin, α-tubulin, Brg-1, Mcl-1, Bax, Bcl-2, Bcl-XL and p53 (Santa Cruz Biotechnology, Santa Cruz, CA); protein light-chain 3 (LC3) (Sigma-Aldrich, St Louis, MO); p62/SQSTM1 (Medical & Biological Laboratories CO., LTD, Japan).

**Microscopy methods**

Total p65 localization as well as binding, internalization and capping of milatuzumab and rituximab were examined by laser scanning confocal microscopy, as described. Cell-cell adhesion was evaluated by visualizing the cells with an inverted microscope (TS100, Nikon) using ×20 PH lens. Further details are available in supplemental materials.

**Evaluation of the in vivo therapeutic activity of rituximab in combination with milatuzumab**

To examine the in vivo activity of rituximab and/or milatuzumab, our previously described preclinical model of human MCL using the SCID mouse was used. Further details are available in supplemental materials.
**Statistical analysis**

Details are available in supplemental materials.

**Results**

Given the variable biology and clinical behavior of MCL, the activity of milatuzumab, rituximab and the combination was tested in six MCL cell lines in order to represent spectrum of this heterogeneous disease. Jeko, Mino, and SP-53 cell lines were used for the mechanistic experiments because of their different histopathologic origins (Jeko, blastoid variant; Mino, SP53, classic MCL) and p53 status. Jeko and SP-53 cells have no detectable mutations, Mino cells have a single mutation at the level of exon 5;27,32,33 p53 protein is present in Mino and SP-53, with Mino expressing the highest level whereas Jeko cells have no detectable p53 (supplemental Figure 1).

**CD20 and CD74 expression on MCL cell lines and patient primary tumor cells.**

CD20 and CD74 antigen expression and MFI were determined by flow cytometry in six MCL cell lines (Figure 1A) and in four primary patient samples (Figure 1B). Expression of CD20 and CD74 surface antigens was observed in all cell lines, although with variable intensity. In order to evaluate the differential expression of CD20 and CD74 between the six MCL cell lines, we determined the number of CD74 and CD20 surface molecules using a flow cytometry-based assay. As shown in Figure 1C, Granta, Jeko and SP-53 expressed the greatest number of CD20 molecules and Mino and HBL-2 the lowest; Mino and SP-53 expressed the highest number of CD74 molecules and Jeko and Rec-1...
the lowest. The number of CD74 molecules was lower in five out of six MCL cell lines when compared to expression of CD20.

**Combination treatment with milatuzumab and rituximab induces enhanced cell death in MCL cell lines and primary patient tumor cells.** Dose titration experiments with Jeko, Mino, and SP53 were performed to determine the optimal dose of milatuzumab. No significant differences in terms of cell death were noticed between 5 and 10 µg/ml at 8, 24 and 48 hours (data not shown) therefore we chose milatuzumab 5 µg/ml for all our *in vitro* experiments. The *in vitro* survival of six MCL cell lines after incubation with milatuzumab, rituximab (10 µg/ml), and combination, in the presence of a cross-linking antibody (in five times excess of binding antibody), was determined at 8, 24, and 48 hours by Annexin-V/PI staining and flow cytometry. As shown in Figure 2A, incubation of six MCL cell lines with cross-linked milatuzumab and rituximab resulted in a statistically significant decrease in cell viability compared to either single agent alone for each of the six cell lines averaged across the three time points examined (P < .01) (mean cell survival averaged across six cell lines and three time points: milatuzumab + rituximab = 17.8±0.9, milatuzumab alone = 41.5 ± 0.9, and rituximab alone = 23.7 ± 1.1). Cell death was rapid, with a significant decrease in viability observed as early as 8 hours. SP-53 and Granta cell lines were the most sensitive to combination treatment. Notably, the Jeko cell line, which represents the blastoid variant of MCL and most resistant phenotype to chemotherapeutic and biological agents,²⁹,³⁰ was the only cell line in where combination treatment resulted in synergistic killing, despite the fact that both milatuzumab and rituximab as single agents showed only modest activity. To determine if the anti-CD20 component was not limited to a rituximab-specific effect, we tested
combination therapy with ofatumumab and veltuzumab, two fully humanized anti-CD20 mAbs that recognize different epitopes on the CD20 protein.\textsuperscript{34,35} As shown in supplemental Figure 2, similar cytotoxic profiles were seen when all three mAbs were used in combination with milatuzumab suggesting that the combination treatment-induced MCL death is not limited to a drug specific effect of rituximab, but rather, generalized anti CD20 signaling. As previously shown in non-MCL lymphoma cell lines,\textsuperscript{22} we confirmed that milatuzumab did not induce cell death in absence of a cross-linking antibody and non-crosslinked rituximab has only minimal effect on MCL cells viability (data not shown).

It was previously reported that milatuzumab-mediated cell death of non-MCL cells did not correlate with antigen density.\textsuperscript{22} However, in MCL cells, statistical analysis showed a significant correlation between CD20/CD74 MFI and cell viability. We observed a statistically significant association between viability of rituximab treated cells and CD20-MFI across all six cell lines, at 8, 24 and 48 hours (supplemental Figure 3, $R^2=0.74$, $P=.028$; $R^2=0.68$, $P=.044$; $R^2=0.66$, $P=.049$). There was also a statistically significant association between viability of milatuzumab treated cells and CD74-MFI, at 24 and 48 hours (supplementary Figure 2, respectively: $R^2=0.79$, $P=.017$; $R^2=0.93$, $P=.002$). In cells treated with both rituximab and milatuzumab, CD20-MFI was significantly associated with percent live cells at 8 hours, but not at 24 and 48 hours (respectively: $R^2=.70$, $P=0.039$; $R^2=0.45$, $P=.15$; $R^2=0.32$, $P=.32$). In cells treated with the combination of rituximab and milatuzumab, CD74-MFI was not significantly associated with cell viability at any time point. These data suggest that anti-tumor responses mediated by single agent rituximab and milatuzumab are related to CD20 and CD74-MFI. In cells
treated with the combination of the two antibodies, responses are not significantly associated with CD74-MFI (respectively: $R^2=0.12$, $P=0.49$; $R^2=0.30$, $P=0.26$; $R^2=0.51$, $P=0.11$).

We next compared the effects of milatuzumab and rituximab, in primary tumor cells from seven MCL patients (clinical characteristics summarized in Figure 2B) after 24 hours of incubation with milatuzumab (5 µg/ml), rituximab (10 µg/ml) or the combination in the presence of a cross-linking antibody. The combination treatment induced an average 79.6% cell death compared to 60% of the milatuzumab-treated cells and 52% of the rituximab-treated cells ($P=0.002$ and $P=0.0001$) (individual patient responses are shown in Figure 2C, representative histograms summarizing patients’ responses are shown in Figure 2D). These data demonstrate combination treatment markedly enhances cell death in MCL cell lines and primary cells regardless of the morphologic variant of MCL compared with single-agent treatment.

**CD74/CD20 aggregation and cytoskeletal integrity are required for milatuzumab and rituximab-induced cell death.** Previous work in non-MCL cell lines showed that cross-linked rituximab induces a redistribution of CD20 molecules on specialized microdomains at the plasma membrane known as lipid rafts. It has also been suggested that membrane rafts may be important in propagating the rituximab mediated pro-death effects. Therefore, we sought to determine if cross-linking of CD20 and CD74 with rituximab and milatuzumab, respectively, formed aggregates within the plasma membrane. Non-immobilized rhodamine-conjugated rituximab treatment (10 µg/ml) of Jeko (Figure 3A), Mino and SP-53 cells (data not shown) induced no change in the
distribution of CD20. As expected, incubation of Jeko (Figure 3A), Mino and SP-53 cells (data not shown) with alexa fluor 488-conjugated milatuzumab (5 µg/ml), in absence of a cross-linking antibody induced rapid internalization of CD74. In contrast, cross-linking of fluorescent rituximab and milatuzumab caused clustering of CD74 and co-clustering with CD20 on Jeko (Figure 3B), Mino, and SP-53 (data not shown) cell surfaces. Representative histograms summarizing the percentage of capped cells in the presence or absence of a cross-linking antibody are shown in Figure 3C (Jeko cells, left panel; Mino cells, right panel). These data clearly show that cross-linking of milatuzumab and rituximab leads to formation and co-localization of large cellular aggregates and capping of CD20 and CD74 proteins on the cell surface and may interrupt the survival signal that has previously been documented with CD74 internalization.\textsuperscript{21}

Cytoskeletal reorganization has been demonstrated to be necessary for CD47,\textsuperscript{38} HLA-DR,\textsuperscript{39} and CD52\textsuperscript{40} cross-linked mAb-mediated cell death in chronic lymphocytic leukemia (CLL) cells. We therefore sought to determine if rituximab and milatuzumab-mediated cell death required CD20/CD74 aggregation and concurrent cytoskeletal association. Figure 4A (Jeko), Figure 4B (Mino) and data not shown (SP-53) demonstrate that pretreatment of MCL cells with latrunculin B (10µM) (and cytochalasin D, 10 µM, data not shown), an agent that prevents actin polymerization, significantly reduced cell aggregation, as assessed by light microscopy 4 hours later. Furthermore, as shown in Figure 4C (Jeko), Figure 4D (Mino) and data not shown (SP-53), pretreatment with latrunculin B significantly reduced the capping and co-localization of CD74 and CD20 antigens (P < .01), as assessed by confocal microscopy 4 hours after the addition of the antibodies.
To further evaluate the link between glycolipid-enriched membrane domain aggregation, alteration of MCL cells cytoskeletal elements, and the transmission of a death signal, we examined the effect of latrunculin B on MCL cells viability. Cells were treated with latrunculin B prior of the addition of immobilized milatuzumab and/or rituximab. After 4 hours incubation, cell viability was determined by Annexin V/PI and flow cytometry. As shown in Figure 4E (Jeko), Figure 4F (Mino) and data not shown (SP-53), pretreatment with lactrunculin B (and cytochalasin D, data not shown) resulted in statistically significant reduced cell death induced by the combination treatment (P < .01 after Holm’s adjustment for multiple comparisons). Our data suggests that co-localization of CD74 and CD20 and cytoskeletal association are necessary events in triggering the milatuzumab/rituximab-mediated cell death signal in MCL cells.

Milatuzumab- and rituximab-mediated cytotoxicity is partially dependent on generation of reactive oxygen species (ROS) and loss of mitochondrial transmembrane potential ($\Delta \Psi_m$)

In order to directly determine if milatuzumab- and rituximab-induced cytotoxicity was mediated by generation of ROS, Jeko (Figure 5A), Mino (Figure 5B) and SP-53 cells (data not shown) were treated with combination rituximab (10 $\mu$g/ml) and milatuzumab (5 $\mu$g/ml), in the presence of a cross-linking antibody, for 0.5, 1, 1.5 and 2 hours, and cells examined for changes in ROS generation using the specific fluorescence probe, dihydroethidine. The combination treatment induced an increase of dihydroethidine fluorescence indicating an increase in ROS generation as early as 0.5 hours, peaking at 1-1.5 hours and reducing at 2 hours, in all the three MCL cell lines examined (Figure 5A,
The levels of ROS in untreated control cells remained unchanged over all incubation time periods. To further explore the implication of ROS in milatuzumab- and rituximab-mediated cell death, Jeko, Mino and SP-53 cells were incubated with N-acetyl cysteine (NAC, 10 mM), a non-specific ROS scavenger, for 1.5 hours, in absence or presence of the combination treatment. NAC was added 15 minutes prior to the addition of the mAbs and, as shown in Figure 5A and 5B (top right panels and data not shown), NAC was able to block ROS generation in treated cells. We next determined cell viability by Annexin V/PI staining and flow cytometry. As shown in Figure 5B (bottom left panel), the addition of NAC led to a statistically significant increase in the viability of Mino cells when compared to milatuzumab- and rituximab-treated cells averaged across 4 and 8 hours incubation (P < .01). Similar results were observed in SP-53 cells (data not shown). In contrast, NAC pretreatment failed to rescue milatuzumab and rituximab treated Jeko cells (P = .064 and P = .054, at 4 and 8 hours respectively) (Figure 5A bottom left panel).

The cytotoxic effect of milatuzumab in combination with rituximab was associated with a gradual and significant loss of \( \Delta \Psi_m \) as early as 8 hours as evidenced by disaggregation of JC-1, in Jeko, Mino and SP-53 cell lines (Figure 5A, 5B bottom right panels, and data not shown). This effect was concurrent with an increase in Annexin binding, indicative of early stages of cell death. The loss in \( \Delta \Psi_m \) caused by the combination treatment was greater than either drug alone with Mino and SP-53 cells and was statistically significant (P< .01 at each of the three time points examined). In Jeko cells, the combination treatment resulted in a statistically significant loss of \( \Delta \Psi_m \) as compared to the rituximab treated cells (P < .01) but not compared to milatuzumab treated cells (P = .079). These
results indicate that ROS generation and mitochondrial membrane dysfunction are involved in the combination treatment-mediated cell death in Mino and SP-53 cells but only partially in Jeko cells. Interestingly, Jeko is the only cell line in which the combination treatment induced a synergistic cell death.

**Milatuzumab and rituximab induce cell death via a non-classical apoptotic mechanism.** Rituximab-induced cell death in B-cell lymphoproliferative disorders has been shown to be associated with the generation of ROS and loss of ΔΨ\(_m\) in a caspase independent fashion.\(^3\) It has recently been shown that milatuzumab induced caspase 2 cleavage in CLL treated cells.\(^2\) We therefore sought to determine if caspase 2 and 3 activation, and PARP cleavage were involved in MCL cell death induced by milatuzumab and rituximab. As shown in supplemental Figure 4, 24 hour incubation of Jeko and Mino cells with milatuzumab (5 µg/ml), rituximab (10 µg/ml) or the combination in the presence of a cross-linking antibody, failed to induce caspase 2 and 3 activation and PARP cleavage. To confirm these findings, we also showed that pretreatment of MCL cell lines with the pan caspase inhibitor, Q-VD-OPH, was able to block cleavage of caspase 2, 3 and PARP (caused by bortezomib) but it had no significant effect on milatuzumab- and rituximab- induced cell death (data not shown).

In order to determine if the milatuzumab- and rituximab-mediated cytotoxicity was due to down-regulation of anti-apoptotic proteins, we tested the effect of this combination treatment on the expression of Bcl-2, Bcl-X\(_L\), and Mcl-1, three critical anti-apoptotic proteins in MCL. As shown in supplemental Figure 5, the expression of Bax, Bcl-2, Bcl-X\(_L\) and Mcl-1 was not altered in response to treatment in Jeko and Mino cells.
Given the absence of an activated classical apoptotic death program in MCL cells treated with milatuzumab and rituximab as evidenced by the absence of caspase 2 and 3 activation and changes in Bcl-2 family members’ expression, we explored alternative mechanisms of cell death. Autophagy describes a physiological mechanism that may serve as a means of temporary survival as is triggered by starvation and metabolic stress. However, if the cellular stress continues, autophagy-mediated cell death may occur.41 Several studies have shown that autophagy plays a major role in the induction of tumor cell death triggered by anti-tumor agents such as tamoxifen42 and arsenic trioxide.43 In order to establish whether the autophagic machinery was involved in milatuzumab- and rituximab-mediated cell death, we examined the effect of this combination treatment on LC3-II and p62 levels. During autophagy, LC3-I is converted to LC3-II through lipidation,44 p62 directly interacts with LC3 and is degradated by the autophagic-lysosome pathway.44 Jeko and Mino cells were incubated for 4 and 24 hours with milatuzumab and rituximab in the presence or absence of chloroquine, which is known to inhibit lysosomal degradation but not autophagosome formation. If milatuzumab and rituximab treatment led to cell death dependent on autophagic mechanisms, then treatment with this combination in presence of chloroquine should result in a significant increase of LC3-II. Treatment with milatuzumab and rituximab in combination with chloroquine did not cause further increase of LC3-II or p62 levels when compared with cells treated with chloroquine alone (supplemental Figure 6 and data not shown). Importantly, MCL cells appear to be relatively resistant to enhanced induction of autophagy caused by rapamycin, a well established reagent that accelerates the autophagic process (supplemental Figure 6). Taken together, these results suggest that
milatuzumab and rituximab treatment of MCL cells is not associated with caspase cleavage, Bcl-2 family member dysregulation or induction of autophagy.

**NF-κB inhibition with immobilized milatuzumab and rituximab in MCL cell lines.**

Previous studies have demonstrated that constitutive activation of NF-κB is an important contributing factor to the pathogenesis of MCL.\(^3,4\) Furthermore, it has already been shown that ligation of CD74 induces CD74 cleavage and internalization and initiates a signaling cascade leading to B-cell proliferation and survival via the NF-κB pathway.\(^2\) Similarly, it has been shown that rituximab is able to inhibit the NF-κB signaling pathway in B-NHL cell lines.\(^4\) We therefore investigated the effects of milatuzumab and rituximab on the NF-κB pathway in MCL cells. We isolated nuclear and cytoplasmic protein fractions from Jeko, Mino, and SP-53 cells after 4 hour incubation with cross-linked milatuzumab, rituximab, or the combination of both. Fractions were analyzed by immunoblot for nuclear p65, α-tubulin (cytoplasmic control) and Brg-1 (nuclear control). As shown in Figure 6 (and data not shown), nuclear p65 levels were not significantly reduced in Jeko, Mino, and SP-53 cells treated with either single agent milatuzumab or rituximab 4 hours after exposure to the respective drug. However, combined exposure to milatuzumab and rituximab resulted in near elimination of nuclear p65 in Mino (Figure 6B) and SP-53 cells (data not shown), while the effect on Jeko cells was less clear (Figure 6A).

To verify these results, we performed the same experiment and evaluated cytosolic and nuclear level of total p65 in Jeko, Mino and SP-53 cells using confocal microscopy. As shown in Figure 6A-B and data not shown, combination treatment with immobilized
milatuzumab and rituximab led to a significant reduction in nuclear p65 compared to single agent milatuzumab and rituximab in Mino and SP-53 cells but, again, the effect was less clear in Jeko cells. These results indicate that disruption of the NF-κB pathway and subsequent loss of the survival signal is induced by the combination treatment in Mino and SP-53 cells and, perhaps, to a lesser degree in Jeko cells.

**In vivo therapeutic activity of combination treatment with milatuzumab and rituximab.** We next evaluated the *in vivo* effect of milatuzumab in combination with rituximab in a preclinical model of human MCL. Six to eight-week old female SCID mice (cb17 scid/scid), NK cells depleted with intra-peritoneal (i.p.) injections of anti-mouse interleukin-2 receptor β monoclonal antibody, were engrafted with 40x10^6 Jeko cells via tail vein injection. Animals in groups of ten, starting at day 15 after engraftment, received trastuzumab (15mg/kg) as a control, rituximab (15mg/kg), milatuzumab (15mg/kg) or the combination of rituximab and milatuzumab, every three days, via i.p. injection. The primary end point of the study was overall survival defined as the time to develop symptoms indicating lethal tumor burden after initiation of treatment. As shown in Figure 7, the mean survival for the combination treated group was 44.5 days (95% CI:39,51), compared to 28 days for trastuzumab treated mice (95% CI:24,30), 33.5 days for the milatuzumab treated mice (95% CI:28,36), and 38 days for the rituximab treated mice (95% CI:36,42). The combination treatment significantly prolonged survival of this group compared to trastuzumab control (P < .0001), milatuzumab (P = .001), and rituximab (P = .03).
Discussion

The natural history of MCL is a course of progressive relapses which are increasingly short-lived as the disease becomes more resistant to therapy. Therefore, the development of new therapeutic options is crucial to improve outcomes for patients with this incurable disease.

Herein, we have shown that the treatment with rituximab and milatuzumab resulted in statistically significant enhanced cell death in all six MCL cell lines and all primary tumor cell samples from seven MCL patients, compared to either agent alone. It has to be noted that the primary cells were obtained from the peripheral blood of MCL patients with leukemic tumor burden. While peripheral blood involvement is present in only a subset of MCL patients and cytotoxicity experiments in such a group may have some limitations, it is important to note that peripheral blood involvement represents an independent negative prognostic factor and is included in the MIPI score.47

We decided on in vitro and in vivo dosing parameters to best reflect concentrations of antibody that were achieved in phase 1 studies. Since there are no published preclinical data on the pharmacokinetics (PK) and pharmacodynamics of milatuzumab, the in vitro and in vivo dosing schedule used in our experiments were based on reported studies in NHLs and multiple myeloma.18,22 Notably, a PK study performed in refractory/relapsed multiple myeloma patients treated with milatuzumab (1.5mg/kg; 4mg/kg; 8mg/kg or 16mg/kg) twice-weekly for 4 weeks showed that at the lowest human dose (1.5mg/kg), the peak levels were 10-20 ug/mL, more than twice the in vitro concentration we used in our study. The dosing in our mice was at 1.2mg/kg human equivalent dose,
approximately the lowest dose used in the clinical study (1.5mg/kg),\textsuperscript{48} perhaps making our findings more clinically relevant.

The combination treatment led to rapid cell death through a death pathway that was caspase independent, non-autophagic and did not involve the Bcl-2 family members. Instead, cell death involved ROS generation, loss of ΔΨₘ, changes in actin dynamics and disruption of the NF-κB pathway.

In contrast with what was previously published by Stein et al. in non-MCL cell lines,\textsuperscript{22} single agent milatuzumab-mediated cell death significantly correlated with antigen density, however this correlation was lost when milatuzumab was combined with rituximab, making this combination an attractive option for a disease with variability of CD74 antigen expression. Notably, the combination treatment induced enhanced cell death in all MCL cell lines and patient samples regardless of the expression of DNA damage sensor genes such as p53 (silenced in Jeko cells and 2 patients samples), ATM (defective in Granta), and cell-cycle checkpoint regulators such as p16 (deleted in REC-1 and Granta).

A large body of literature supports the association between CD20, lipid rafts, cytoskeletal elements and initiation of cell death.\textsuperscript{37,49,50} However, little is known about the cytoskeletal associations that might control the aggregation of CD74. Here we showed that immobilized milatuzumab and rituximab led to capping of antibody-antigen complexes on the majority of the MCL cells examined. Unlike rituximab, non-immobilized milatuzumab induced quick internalization of the receptor leading us to hypothesize that the clustering of CD74 on the cell surface by immobilized milatuzumab may mediate downstream pathways that trigger a death signal. Also, the co-localization of CD74 and
CD20 in cross-linked antibody-treated cells may promote an amplification of the death
signal as seen by other studies.\textsuperscript{37,49} To further characterize the CD74 and CD20 capping
and to explain the receptor redistribution through a linkage to the cytoskeleton, we
demonstrated that pretreatment with actin polymerization inhibitors such as latrunculin B
and cytochalasin D significantly reduced cross-linked-antibody-mediated capping, cell-
cell adhesion, and cell death in MCL cells treated with the combination of milatuzumab
and rituximab and a cross-linking antibody.

Previous work in non-MCL cell lines has determined that rituximab-induced cell death
was caspase independent\textsuperscript{31} which was confirmed in our analysis. Stein \textit{et al.}\textsuperscript{22} showed
that milatuzumab-mediated cell death in Burkitt lymphoma cell lines was dependent upon
caspase 3 and 8 activation. More recently, caspase 2 cleavage was observed in CLL cells
treated with milatuzumab.\textsuperscript{24} Here, we established that the engagement of CD20 and
CD74 resulted in ROS generation and loss of $\Delta \Psi_m$ but cell death occurred in absence of
caspase activation or alteration of Bcl-2 family member expression.

Prior studies have demonstrated that constitutive activation of NF-kB is a contributing
factor to the pathogenesis of MCL.\textsuperscript{3,45} It has also been previously shown in non-MCL cell
lines that rituximab disrupts the NF-kB pathway and sensitizes tumor cells to
chemotherapeutic agents, in part by selectively down-modulating Bcl-2 and Bcl-X\textsubscript{L}.\textsuperscript{46}
Ligation of CD74 by its natural ligand MIF also resulted in NF-\kappaB activation through the
PI3K/Akt survival pathway and via Syk phosphorylation.\textsuperscript{51,52} Here we showed that the
combination treatment was able to disrupt the NF-\kappaB pathway by inhibiting the
translocation of p65 from the cytosol to the nucleus. However we failed to show down-
modulation of NF-\kappaB targets such as Bcl-2 and Bcl-X\textsubscript{L}.\textsuperscript{3} Interestingly, Starlets \textit{et al.},\textsuperscript{21}
showed that CD74 stimulation of CLL cells initiates a cascade of events that leads to proliferation and survival through activation of the NF-kB pathway and up-regulation of Bcl-X\textsubscript{L}, however only up-regulation of the transcript was shown with no evaluation of protein levels.

Based on these findings, it is plausible that the initial and common event in MCL cell death mediated by rituximab and milatuzumab could be the patching/capping on cell surface that subsequently triggers ROS generation, loss of $\Delta \Psi_m$ and involves the underlying actin cytoskeleton rearrangements. Our results also suggest that disruption of the NF-kB pathway, through inhibition of p65 nuclear translocation, may contribute to milatuzumab- and rituximab-induced cell death.

The intriguing results obtained with the \textit{in vitro} experiments led us to investigate the combination of milatuzumab and rituximab in our recently described \textit{in vivo} model of MCL\textsuperscript{30}. The Jeko model was used as it represents the most aggressive and stringent preclinical model to evaluate potential experimental therapeutic strategies in MCL. Importantly, the combination of milatuzumab and rituximab significantly prolonged survival compared to trastuzumab treated controls ($P < .0001$) but also to single agent milatuzumab ($P = .001$) and single agent rituximab ($P = .03$).

In summary, milatuzumab and rituximab combination therapy resulted in \textit{in vitro} enhanced cell death in MCL cell lines and primary tumor samples, regardless of antigen density and p53 status. Furthermore, enhanced survival was observed with combination therapy in an \textit{in vivo} murine model of MCL. To the best of our knowledge, our findings are the first to show that milatuzumab and rituximab could potentially represent an active therapeutic strategy for the treatment of MCL patients. Our data supports the clinical
development of milatuzumab and rituximab in MCL, and based on these collective findings, a phase I/II study is currently underway at our institution evaluating combination milatuzumab and veltuzumab in B-NHL (NCT00989586).

**Acknowledgements**

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**Author Contributions**

L.A.: designed and performed research, analyzed data, wrote and reviewed the paper, approved the final version of the manuscript;

B.Y.: performed research, analyzed data, approved the final version of the manuscript;

B.C.: performed research, analyzed data, reviewed drafts and approved the final version of the manuscript;

F.Y.: performed research and approved the final version of the manuscript;

J.S.: performed research and approved the final version of the manuscript;

R.L.: performed research and approved the final version of the manuscript;

E.H.: performed research and approved the final version of the manuscript;

M.E.L.: performed statistical analysis, approved the final version of the manuscript;

X.L.: performed statistical analysis, approved the final version of the manuscript;
C.Q.: performed research, analyzed data and approved the final version of the manuscript;
G.L.: performed research, analyzed data and approved the final version of the manuscript;
N.M.: designed research, reviewed drafts and approved the final version of the manuscript;
M.P-I.: performed research, analyzed data, reviewed drafts and approved the final version of the manuscript;
O.A.O.: designed research, provided cell lines, reviewed drafts and approved the final version of the manuscript;
D.M.G.: provided reagent, reviewed drafts and approved the final version of the manuscript;
J.C.B.: designed research, reviewed drafts and approved the final version of the manuscript;
K.A.B.: provided samples, reviewed drafts and approved the final version of the manuscript;
R.A.B.: designed and supervised research, obtained funding for the research work, reviewed drafts and approved the final version of the manuscript.

**Disclosure of Conflict of Interest**

D.M.G is an officer and member of the Board of Directors of Immunomedics, Inc., which owns milatuzumab. The remaining authors have no relevant conflict of interest to disclose.
References


Table 1. Characteristics of Mantle Cell Lymphoma Cell Lines

<table>
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<tr>
<th></th>
<th>Jeko</th>
<th>Mino</th>
<th>SP-53</th>
<th>Rec-1</th>
<th>HBL-2</th>
<th>Granta</th>
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<tr>
<td>Doubling time (hours)</td>
<td>36-48</td>
<td>48</td>
<td>48-72</td>
<td>38</td>
<td>48</td>
<td>48-72</td>
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<tr>
<td>Epstein-Barr virus</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cyclin D1 expression</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Evidence of t(11;14)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p53 expression</td>
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Figure Legends

Figure 1. CD20 and CD74 expression on MCL cell lines and primary MCL tumor cells. Representative histograms and tables showing CD20 and CD74 mean fluorescence intensity and percent positive cells based on comparison to isotype control in six MCL cell lines (Figure 1A) and primary cells from four different MCL patients (Figure 1B). Figure 1C shows the number of CD20 and CD74 molecules per cell in six MCL cell lines.

Figure 2. Immobilized rituximab and milatuzumab treatment-induced cell death in MCL cells. Six MCL cell lines (A) and primary cells from seven patients (C, D; patient characteristics are summarized in panel B) were treated with rituximab (10 µg/ml) and/or milatuzumab (5 µg/ml), + cross-linking antibody. Cell death was determined by Annexin V/PI staining and flow cytometry at 8, 24 and 48 hours for the six cell lines and at 24 hours for MCL primary cells. Data are shown as percent of Annexin V-/PI- cells (live cells) and are normalized to untreated control. Individual patient responses are shown in Figure 2C, representative histograms summarizing patient responses are shown in Figure 2D.

Combination treatment resulted in statistically significant enhanced induction of MCL cell death compared to either agent alone (P < .01).

Figure 3. CD20 and CD74 labeling by specific fluorescent antibodies. Binding and internalization of CD20 and CD74 in Jeko cells were examined by laser scanning confocal microscopy. Jeko cells were incubated with rhodamine-conjugated rituximab
(red) and alexa fluor 488-conjugated milatuzumab (green) in the absence (Figure 3A) or presence (Figure 3B) of a cross-linking antibody, for 2 hours at 37°C. DRAQ5™ was used for nuclear staining (blue). Cross-linked milatuzumab forms large aggregates and co-localizes with crosslinked rituximab on the surface of MCL cells. Representative histograms summarizing the percentage of capped cells in the presence or absence of a cross-linking antibody are shown in Figure 3C (Jeko cells, left panel; Mino cells, right panel). At least 100 cells/condition were counted for each of the three independent experiments. Anti-Fc: anti-Fc cross-linking antibody.

Figure 4. Cell-cell interactions and cytoskeleton organization are involved in MCL cell death evoked by immobilized rituximab and milatuzumab

Jeko (Figure 4A) and Mino (Figure 4B) cells were treated with DMSO or the actin polymerization inhibitor, latrunculin B (10 µM), for 45 minutes, prior the addition of rituximab and milatuzumab, + cross-linking antibody. Cell aggregation was assessed 4 hours later by light microscopy. Pretreatment with latrunculin B significantly reduced cell aggregation.

Jeko (Figure 4C) and Mino (Figure 4D) cells were treated with DMSO or an actin polymerization inhibitor (latrunculin B), for 45 minutes, prior the addition of rituximab and milatuzumab, + a rhodamine-conjugated cross-linking antibody. The formation of aggregates and caps on Jeko (Figure 4C, left panel) and Mino (Figure 4D, left panel) cells’ surface was evaluated 4 hours later by confocal microscopy. Representative histograms summarizing the percentage of capped cells in the presence or absence of latrunculin B are shown in Figure 4C (Jeko cells, right panel) and 4D (Mino cells, right panel).
At least 100 cells/condition were counted for each of the three independent experiments. Pretreatment with latrunculin B significantly (P < .01) reduced the capping and co-localization of CD74 and CD20 antigens.

Cell death evaluation of Jeko (Figure 4E) and Mino (Figure 4F) cells treated with DMSO or latrunculin B, for 45 minutes, prior the addition of rituximab and milatuzumab + cross-linking antibody. Cell death was determined 4 hours later by Annexin-V/PI and flow cytometry. Pretreatment with lactrunculin B resulted in statistically significant reduced cell death induced by the combination treatment (P < .01).

**Figure 5. Rituximab and milatuzumab cytotoxicity is partially dependent on ROS generation and loss of mitochondrial membrane potential (ΔΨm).**

Jeko (Figure 5A, top left panel) and Mino (Figure 5B, top left panel) cells were treated with rituximab and milatuzumab + cross-linking antibody for the indicated timepoints. ROS generation was determined by flow cytometry analysis using dihydroethidine dye. ROS generation is indicated by right shift of the dihydroethidine curves. Hydrogen peroxide was used as positive control.

Figure 5A and Figure 5B, top right panel, show pretreatment of MCL cells with the non-specific ROS scavenger N-acetyl cysteine (NAC, 10 mM) and inhibition of ROS generation induced by antibodies treatment.

For rescue experiments, Jeko (Figure 5A, bottom left panel) and Mino (Figure 5B, bottom left panel) cells were incubated with NAC for 4 and 8 hours in absence or presence of the antibodies, and cell viability determined by Annexin V/PI staining and flow cytometry.
Figure 5A (bottom right panel) and Figure 5B (bottom right panel) show mitochondrial membrane potential ($\Delta \Psi_m$) changes in Jeko and Mino cells treated with rituximab, milatuzumab or the combination of both, in the presence of a cross-linking antibody, for 4, 8 and 24 hours. $\Delta \Psi_m$ changes were quantified by flow cytometry determination using JC-1. Data are shown as the percentage treated cells with intact mitochondria relative to untreated cells at the same time points.

**Figure 6.** Rituximab and milatuzumab combination treatment inhibited nuclear total p65 translocation in MCL cell lines. Top panels: nuclear and cytosolic protein fractions collected at 4 hours were prepared from Jeko (Figure 6A) and Mino (Figure 6B) cell lines and subjected to immunoblot analysis for p65, $\alpha$-tubulin (cytoplasmic control) and Brg-1 (nuclear control). Bottom panels: confocal microscopic analysis of intracellular localization of total p65 (green) in Jeko (Figure 6A) and Mino (Figure 6B) cells. MCL cells were treated with cross-linking antibody, trastuzumab, rituximab, milatuzumab and the combination of rituximab and milatuzumab, in the presence of a cross-linking antibody. After 4 hours cells were collected and prepared for confocal microscopy analysis. DRAQ5$^\text{TM}$ was used for nuclear staining.

**Figure 7.** Evaluation of in vivo therapeutic activity of rituximab and milatuzumab in the preclinical MCL model. SCID mice (in groups of ten) were injected i.v with $40 \times 10^6$ JeKo cells and observed daily for signs of tumor burden. mAbs (trastuzumab, 15mg/kg, rituximab, 15mg/kg, milatuzumab, 15mg/kg, or the combination of rituximab and milatuzumab) were given every three days, via i.p. injection, starting at day 15 after
engraftment. The mean survival for rituximab and milatuzumab treated mice was 44.5 days (95% CI:39.51), compared to 33.5 days for the milatuzumab treated mice (95% CI:28.36), and 38 days for the rituximab treated mice (95% CI:36.42).
### Figure 1A

#### CD20

<table>
<thead>
<tr>
<th></th>
<th>Isotype (IgG1) ± SD (%)</th>
<th>CD20 % positive cells</th>
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<tbody>
<tr>
<td>Jeko</td>
<td>0.0 ± 0.0</td>
<td>97.7 ± 0.01</td>
</tr>
<tr>
<td>Mino</td>
<td>0.0 ± 0.0</td>
<td>89.4 ± 0.05</td>
</tr>
<tr>
<td>SP-53</td>
<td>0.0 ± 0.0</td>
<td>98.0 ± 0.01</td>
</tr>
<tr>
<td>Rec-1</td>
<td>0.1 ± 0.0006</td>
<td>86.5 ± 0.12</td>
</tr>
<tr>
<td>HBL-2</td>
<td>0.0 ± 0.0</td>
<td>66.1 ± 0.11</td>
</tr>
<tr>
<td>Granta</td>
<td>0.0 ± 0.0</td>
<td>99.5 ± 0.002</td>
</tr>
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#### CD74

<table>
<thead>
<tr>
<th></th>
<th>Isotype (IgG2a) ± SD (%)</th>
<th>CD74 % positive cells</th>
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<tbody>
<tr>
<td>Jeko</td>
<td>0.9 ± 0.006</td>
<td>92.5 ± 0.01</td>
</tr>
<tr>
<td>Mino</td>
<td>1.9 ± 0.02</td>
<td>99.2 ± 0.0005</td>
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<tr>
<td>SP-53</td>
<td>0.7 ± 0.003</td>
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<tr>
<td>Rec-1</td>
<td>0.6 ± 0.002</td>
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<td>HBL-2</td>
<td>0.8 ± 0.004</td>
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<td>Granta</td>
<td>1.3 ± 0.001</td>
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### Figure 1B

#### CD20

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<td>Patient 3</td>
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<td>Patient 4</td>
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#### CD74

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<tr>
<td>Patient 3</td>
<td>1.72</td>
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<td>Patient 4</td>
<td>2.77</td>
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Figure 1C

**CD20**

**CD74**

![Bar charts showing the number of molecules for CD20 and CD74 in different cell lines: Jeko, Mino, SP-53, Rec-1, HBL-2, and Granta. The x-axis represents the cell lines, and the y-axis represents the number of molecules.](image-url)
Figure 2A

% Live cells (Ann-V/PI-cells) normalized to untreated control

Jeko  Minio  SP-53
Control  Hercep  Ritux  Milat  Combo
Control  Hercep  Ritux  Milat  Combo
Control  Hercep  Ritux  Milat  Combo

Rec-1  HBL-2  Granta
Control  Hercep  Ritux  Milat  Combo
Control  Hercep  Ritux  Milat  Combo
Control  Hercep  Ritux  Milat  Combo

8 hours  24 hours  48 hours
### Figure 2B

<table>
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<tr>
<th>Patient</th>
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<th>Stage</th>
<th>Previous therapy</th>
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<tr>
<td>1</td>
<td>52 M</td>
<td>Blastoid</td>
<td>IVB</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>72 F</td>
<td>Blastoid</td>
<td>IVB</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>65 M</td>
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<td>IVB</td>
<td>No</td>
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<tr>
<td>4</td>
<td>59 M</td>
<td>Classic</td>
<td>IVA</td>
<td>No</td>
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<tr>
<td>5</td>
<td>71 M</td>
<td>Classic</td>
<td>IVB</td>
<td>Yes (including rituximab)</td>
</tr>
<tr>
<td>6</td>
<td>63 F</td>
<td>Blastoid</td>
<td>IVB</td>
<td>Yes (including rituximab)</td>
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<tr>
<td>7</td>
<td>67 F</td>
<td>Classic</td>
<td>IVB</td>
<td>Yes (including rituximab)</td>
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Figure 2C

Graph showing the percentage of live cells (Annexin-PI-cells) normalized to untreated control across different treatments for different patients.

- **X-axis:** Control, Rituximab, Milatuzumab, Combination
- **Y-axis:** % Live cells (Annexin-PI-cells) normalized to untreated control

Legend:
- Patient 1
- Patient 2
- Patient 3
- Patient 4
- Patient 5
- Patient 6
- Patient 7
- Mean
Figure 2D

% Live cells (Ann-/PI- cells) Normalized to untreated control

- Control
- Rituximab
- Milatuzumab
- Combination

N = 7

*
Figure 3B

Milatuzumab + anti-Fc

Rituximab + anti-Fc

DRAQ5™

Overlay
Figure 3C

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- on November 12, 2017.
- by guest

www.bloodjournal.org
Figure 4A

Anti-Fc

- latrunculin B

+ latrunculin B

Combo + anti-Fc

100μm
Figure 4C
Figure 4D

- latrunculin B

- latrunculin B

% Capped Cells

P < .01

- latrunculin B

+ latrunculin B
Figure 4F
**Figure 5B**

The figure shows the results of a study comparing different treatments on cell viability and mitochondrial integrity. The left panel displays flow cytometry data for dihydroethidium (DHE) staining, with histograms for different conditions: Untreated control, Combination 0.5h, Combination 1h, Combination 2h, and Positive control. The x-axis represents DHE fluorescence, and the y-axis represents the number of events.

The right panel also shows flow cytometry data with similar conditions, with an additional category for Combination + NAC.

The bottom left graph illustrates the percentage of live cells (Annexin-V negative) normalized to untreated controls, showing a decrease in live cells with treatment over time (4 hours vs 8 hours).

The bottom right graph represents the relative percentage of intact mitochondria for different treatments and time points (8 hours, 24 hours, and 48 hours), with asterisks indicating statistically significant differences.
Figure 6A

**Jeko**

<table>
<thead>
<tr>
<th></th>
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<th>Milatuzumab</th>
<th>Combo</th>
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<tbody>
<tr>
<td>Total p65</td>
<td>Cit</td>
<td>Nucl</td>
<td>Cit</td>
<td>Nucl</td>
</tr>
<tr>
<td>Brg-1</td>
<td>Cit</td>
<td>Nucl</td>
<td>Cit</td>
<td>Nucl</td>
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<tr>
<td>α-Tubulin</td>
<td>Cit</td>
<td>Nucl</td>
<td>Cit</td>
<td>Nucl</td>
</tr>
</tbody>
</table>

**Untreated Control**

![Untreated Control Images](image)

**Crosslinker**

![Crosslinker Images](image)

**Trastuzumab**

![Trastuzumab Images](image)
Figure 6B

Mino

<table>
<thead>
<tr>
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<td>α-Tubulin</td>
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<td>Nucl</td>
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<table>
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<tr>
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<td>DRAQ5</td>
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Rituximab         | Milatuzumab | Combination |
Figure 7

- Control (10 mice)
- Rituximab (10 mice)
- Milatuzumab (10 mice)
- Milatuzumab + Rituximab (10 mice)

Survival rates and statistical significance:
- Control vs Milatuzumab + Rituximab: P < .0001
- Milatuzumab vs Rituximab: P = .001
- Rituximab vs Milatuzumab + Rituximab: P = .03
Combination anti-CD74 (milatuzumab) and anti-CD20 (rituximab) monoclonal antibody therapy has \textit{in vitro} and \textit{in vivo} activity in mantle cell lymphoma

Lapo Alinari, Bo Yu, Beth A. Christian, Fengting Yan, Jungook Shin, Rosa Lapalombella, Erin Hertlein, Mark E. Lustberg, Carl Quinion, Xiaoli Zhang, Gerard Lozanski, Natarajan Muthusamy, Mette Praetorius-Ibba, Owen A. O’Connor, David M. Goldenberg, John C. Byrd, Kristie A. Blum and Robert A. Baiocchi