Molecular Pharmacology Fast Forward. Published on June 18, 2014 as DOI: 10.1124/mol.114.092684
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MOL Manuscript #92684

Analyses of CD20 Monoclonal Antibody-Mediated Tumor Cell Killing Mechanisms: Rational Design of Dosing Strategies

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Running Title: Optimizing Doses for Monoclonal Antibodies in Cancer

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Text pages: 8

Figures: 2

References: 79

Abstract: 250 words

Introduction: 656 words

Discussion (remainder of text): 3242 words

Abbreviations used: CLL, chronic lymphocytic leukemia; OBZ, obinutuzumab; OFA, ofatumumab; RTX, rituximab

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Abstract

Since approval of rituximab for treatment of B-cell non-Hodgkin's lymphoma, development of monoclonal antibodies (mAbs) for cancer treatment and elucidation of their cytotoxic mechanisms have been subject to intense investigations. Compelling evidence indicates that rituximab and another CD20 mAb, ofatumumab, must utilize the body's cellular and humoral immune effector functions to kill malignant cells. Other FDA-approved mAbs, including obinutuzumab, cetuximab and trastuzumab, require, in part, these effector mechanisms to eliminate tumor cells. Although gram quantities of mAbs can be administered to patients, our investigations of CD20 mAb-based therapies for chronic lymphocytic leukemia (CLL), including correlative measurements in clinical trials and studies with primary cells and cell lines, indicate that if tumor burdens are high, effector mechanisms necessary for mAb activity can be saturated or exhausted, thus substantially compromising the efficacy of high dose mAb therapy. Under these conditions another reaction, trogocytosis, predominates, in which bound CD20 mAb and CD20 are removed from targeted cells by effector cells that express Fcγ receptors, thereby allowing malignant cells to escape unharmed and continue to promote disease pathology. To address this problem, we propose that a low-dose strategy, based on administering 30-50 mg of CD20 mAb three times per week, may be far more effective for CLL than standard dosing because it will minimize effector function saturation and reduce trogocytosis. This approach may have general applicability to other mAbs that utilize immune effector functions, and could be formulated into a subcutaneous treatment strategy that would be more accessible and possibly more efficacious for patients.

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Introduction

There is a voluminous literature that documents the successful use of monoclonal antibodies (mAbs) in the immunotherapy of cancer (Scott *et al.*, 2012; Mahalingam and Curiel, 2013; Zigler *et al.*, 2013; Sliwkowski and Mellman, 2013). However, although numerous clinical investigations have demonstrated varying degrees of efficacy of a given mAb (alone or in combination with chemotherapy) there remains considerable uncertainty with respect to which mechanisms promote tumor cell elimination in humans (Glennie *et al.*, 2007; Boross and Leusen, 2012; Zigler *et al.*, 2013; Sliwkowski and Mellman, 2013). Studies in mouse models have provided insight, but may be model-dependent, favoring one mechanism over another, based simply on the details of the model design (Taylor and Lindorfer, 2014). Perhaps the greatest controversy centers on distinguishing between *direct cytotoxic effects* of a mAb on tumor cells and/or their environment, versus establishing an absolute requirement of the mAb to harness one or more of the body's immune effector mechanisms to kill tumor cells. For example, based only on in vitro experiments with cell lines, binding of the CD20 mAbs rituximab (RTX), ofatumumab (OFA) and obinutuzumab (OBZ) to B cells may initiate signaling cascades that mediate cell killing directly by pathways that include apoptosis as well as, in the case of OBZ, a non-caspase dependent lysosomal reaction pathway (Glennie *et al.*, 2007; Mossner *et al.*, 2010; Alduaji *et al.*, 2011). However, increasing evidence, based on rigorous experiments with primary tumor cells, carefully controlled murine model studies, and correlative measurements in clinical trials, has clearly demonstrated that the most important cytotoxic mechanisms of these mAbs require immune effector functions (Gong *et al.*, 2005; Glennie *et al.*, 2007; Wilson *et al.*, 2011; Beurskens *et al.*, 2012; Golay and Introna, 2012; Golay *et al.*, 2013a; Montalvao

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et al., 2013; Bologna *et al.*, 2013). That is, tumor cells that are opsonized with CD20 mAbs are killed by cellular effector reactions which include antibody dependent cell mediated cytotoxicity (ADCC), phagocytosis by macrophages and possibly neutrophils, or by complement dependent cytoxtoxicity (CDC).

Because these effector functions are absolutely required for CD20 mAb efficacy, we submit that the usual pharmacological concepts of maximum tolerated dose and dose-limiting toxicity, axiomatic for evaluation of chemotherapeutic agents for cancer treatment, do not obtain for use of these mAbs. Indeed, although the pharmacokinetics and pharmacodynamics of RTX and of OFA for high mAb doses have been intensively studied (Berinstein *et al.*, 1998; Coiffier *et al.*, 2010; Golay *et al.*, 2013b), several lines of evidence indicate that, particularly for chronic lymphocytic leukemia (CLL), the most effective doses, and their timing, require critical re-evaluation (Lindorfer *et al.*, 2012; Baig *et al.*, 2014; Zent *et al.*, 2014).

RTX, the first mAb approved for treatment of cancer, has proven quite successful in the treatment of B-cell lymphomas (McLaughlin *et al.*, 1998; Davis *et al.*, 2000; Cheson and Leonard, 2008; Weiner, 2010). Indeed, when combined with chemotherapy, the usual 375 mg/m^2 dose of RTX has been found to provide substantial therapeutic benefit for a number of indications, including CLL (Hallek *et al.*, 2010; Furman *et al.*, 2014). Therefore, a considerable research effort has been devoted to understanding the cytotoxic mechanisms of RTX as well as its limitations in order to develop second and third generation CD20 mAbs designed to have enhanced clinical activity (Teeling *et al.*, 2004; Cheson, 2010; Mossner *et al.*, 2010; Peipp *et al.*, 2011; Alduaji *et al.*, 2011). Recent provocative evidence indicates that other FDA-approved

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mAbs, including cetuximab (anti-epidermal growth factor receptor), ipilimumab (anti-cytotoxic T lymphocyte associated antigen 4) and trastuzumab (anti-human growth factor receptor 2, HER2), also directly or indirectly make use of effector mechanisms mediated by cells that express Fcγ receptors (Zhang *et al.*, 2007; Musolino *et al.*, 2008; Taylor *et al.*, 2009; Botta *et al.*, 2012; Bulliard *et al.*, 2013; Kim and Ashkenazi, 2013; Simpson *et al.*, 2013; Bianchini and Gianni, 2014). Therefore the lessons learned based on analyses of CD20 mAbs may have general implications for these mAbs as well.

Correlative Studies Associated with CD20 mAb Treatment of Chronic Lymphocytic Leukemia (CLL)

Ten years ago we first reported results of correlative studies based on analyses of blood samples drawn from patients with CLL who were being treated with the standard weekly doses of 375 mg/m^2 of RTX (Kennedy *et al.*, 2004). These results have been replicated several times and thus provide a framework for understanding key issues that underlie use of unconjugated mAbs in cancer immunotherapy. We found that after infusion of *only 30 mg* of RTX, approximately 70% of the circulating CLL cells present before infusion were removed from the circulation, principally due to clearance of the RTX-opsonized cells by fixed tissue macrophages in the liver and spleen (Schreiber and Frank, 1972; Atkinson and Frank, 1974; Montalvao *et al.*, 2013). Surviving circulating CLL cells were also opsonized with inactive complement fragment C3d. Based on comparable studies with antibody-opsonized erythrocytes, the clearance mechanism may have been mediated in part synergistically by Fcγ receptors and complement receptors on macrophages (Schreiber and Frank, 1972; Atkinson and Frank, 1974; Lindorfer *et al.*, 2014). Immediately after completion of the full RTX

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infusions (600-700 mg), circulating CLL cell levels had *increased considerably* (relative to the levels after infusion of only 30 mg), due to re-equilibration of a "second wave" of cells from other compartments, and these cells persisted in the bloodstream despite high plasma levels of RTX (~100 µg/ml). A key clue to understanding why these cells were not cleared from the circulation was revealed when we found that CD20 expression on these "surviving" CLL cells was substantially reduced, in most cases approximately 20-fold. In addition, in several patients complement titers were also reduced 10-fold or more. This was the first observation of what we have characterized as the "perfect storm" that occurs when large doses of CD20 mAbs are infused in CLL patients with high burdens of circulating malignant cells. Under these conditions, after an initial very rapid clearance of a large fraction of circulating cells, the surviving CLL cells are no longer subject to attack or clearance, despite the presence of large amounts of the CD20 mAb in the bloodstream. These cells have very low levels of CD20, and the low CD20 levels persist for several days to weeks, due to the continued presence of the mAb in the circulation (Beurskens *et al.*, 2012; Baig *et al.*, 2014). In addition, for some period of time, an important effector function, complement, is exhausted (Kennedy *et al.*, 2004; Beurskens *et al.*, 2012). Moreover, as we have recently reported in a second observational study of OFA immunotherapy, cells that are isolated from the bloodstream soon after mAb infusion are no longer subject to CDC, even in the presence of fresh serum and additional CD20 mAb, presumably because CD20 levels are so low (Baig *et al.*, 2014). These observations have been replicated on more than 60 CLL patients in several clinical studies, conducted at the University of

Virginia, the NIH, and the Mayo Clinic (Kennedy *et al.*, 2004; Williams *et al.*, 2006; Beurskens *et al.*, 2012; Baig *et al.*, 2014; Zent *et al.*, 2014).

Trogocytosis of mAb-Opsonized Cells

CD20 is expressed at quite comparable levels on CLL cells in the bloodstream and in other compartments (Tam *et al.*, 2008). We have concluded, based on comprehensive in vitro experiments as well as a mouse model, that the second wave of cells that re-equilibrates into the bloodstream, as well as cells not cleared in the early phase of the CD20 mAb infusion, rapidly lose CD20 due to trogocytosis or "shaving" (Beum *et al.*, 2006), which predominates after natural clearance mechanisms are saturated or exhausted.

Trogocytosisis is mediated by acceptor cells which express Fcγ receptors, including macrophages, monocytes, NK cells, and neutrophils (Beum *et al.*, 2006; Li *et al.*, 2007; Beum *et al.*, 2008a; Beum *et al.*, 2011). During trogocytosis the mAb-opsonized target donor cell and the acceptor cell first form an immunologic synapse, due to binding of Fcγ receptors on the acceptor cells to cognate Fc sites on the "immune complexed" mAb bound to CD20 on the opsonized B cells (Joly and Hudrisier, 2003; Taylor, 2013; Rossi *et al.*, 2013). The acceptor cell then removes the mAb/CD20 immune complex from the opsonized B cell along with a portion of the plasma membrane, and ultimately internalizes the immune complex. The reaction is rapid; the process goes to completion in less than one hour. At first examination, this reaction appears to be antithetical to Metchnikoff's definition of macrophages as "big eaters", which should engage in phagocytosis (Taylor, 2013). However, our in vitro experiments

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indicate that macrophages are capable of executing *both* processes, when presented with RTX-opsonized cells (**Figure 1,** (Daubeuf *et al.*, 2010)). That is, in certain cases the macrophages are true to their phenotype and completely internalize opsonized B cells, but in other cases the macrophages simply remove and internalize CD20 and RTX. We confirmed, by flow cytometry, that the recovered B cells had indeed lost substantial amounts of bound RTX and CD20, but were otherwise intact (Daubeuf *et al.*, 2010).

The results of these experiments place into context our pilot clinical study in which lower doses of RTX were infused in CLL patients (Williams *et al.*, 2006). We reasoned that thrice weekly iv RTX doses of only 20 mg/m^2 should provide enough mAb to target and clear circulating cells, but that the small dose of RTX would minimize its concentration in the bloodstream afterwards. Therefore, these low doses should better preserve effector functions, reduce CD20 loss via trogocytosis, and allow for more rapid re-expression of CD20 on CLL cells, thus making possible additional targeting and clearance of cells following subsequent low-dose RTX infusions. The general paradigm was validated in that study and was recently confirmed (Zent *et al.*, 2014). During each infusion, targeted CLL cells are cleared very quickly, supporting the concept that the clearance mechanism follows the same pattern reported by Frank's group for clearance of IgG-opsonized erythrocytes (Atkinson and Frank, 1974; Schreiber and Frank, 1972). We also found that B cell clearance and trogocytosis of CD20 occurred simultaneously. The most reasonable explanation is that as RTX-opsonized cells come into contact with fixed tissue macrophages in the liver and spleen, some cells are removed and phagocytosed, whereas others are partially shaved, and return back into the

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bloodstream. However, as these cells still have bound IgG RTX, they can be cleared in the second or third or even later passes through these organs.

The Importance of exhaustion

It is clear that very large quantities (~2 grams) of immunotherapeutic mAbs such as RTX or OFA can be infused iv in patients, because for the most part there is no dose-limiting toxicity. However, at high B cell burdens in CLL, high mAb doses generate very large quantities of "immune complexes" (mAb-opsonized cells) which can not only activate and exhaust complement, but also can overwhelm and saturate cell-mediated effector functions. One of these is phagocytosis and/or direct killing of CD20 mAb-opsonized cells by macrophages. Several well-designed mouse models have clearly demonstrated the importance of this cytotoxic mechanism, and have provided evidence for saturation or exhaustion. Leusen's group has examined how low and high tumor burdens are handled in a peritoneal syngeneic mouse model (Boross *et al.*, 2011). They find that at low cell burdens, complement is adequate to clear the cells, but at 10-fold higher cell burdens both complement and macrophage-mediated killing and clearance are required. However, even though 10-fold more mAb is administered at the higher cell burdens, thus maintaining the same mAb/tumor ratio, the percentage of cells cleared drops from 95% to 70%. That is, for both challenges there is adequate mAb to easily saturate the cells with anti-CD20 mAb, but at the higher tumor burdens the effector mechanisms simply cannot adequately process and destroy the large number of immune-complexed, mAb-opsonized cells. These observations are re-inforced by in vitro studies which indicate that a monocyte-derived human macrophage can

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phagocytose no more than 10 RTX-opsonized CLL cells (Dr. Clive Zent, University of Rochester Medical Center, personal communication). The macrophage cannot take up any more RTX-opsonized cells for at least 24 hours, until the ingested cells are processed and degraded. The human liver has approximately 3 X 10^{10} Kupffer cells (macrophages) (Boyer, 2003)**.** Given the high circulating cell burdens common in CLL (100,000 cells/μL), thus corresponding to about 4 X 10^{11} malignant B cells, clearance of 80-90% of these mAb-opsonized cells by liver macrophages presents a real challenge. In addition, malignant cells will rapidly re-equilibrate from other compartments. Therefore, it is not surprising that following infusions of even large quantities of RTX or OFA, the cell counts drop precipitously, but then increase over 24 hours, even though the mAb remains at high concentrations in the bloodstream.

Similarly, ADCC of CD20 mAb-opsonized cells mediated by NK cells can also be exhausted at high cell burdens. Berdeja et al. reported that one hour after treatment of lymphoma patients with large doses of RTX, the ADCC activity of their NK cells against RTX-opsonized targets was substantially reduced, but was partially restored after 24 hrs (Berdeja *et al.*, 2007). This clinical observation of NK cell exhaustion is complemented by several in vitro investigations. Bhat and Watzl reported that after NK cells had killed two to four substrates, the levels of perforin and granzyme in the cells had decreased, and the killing capacity of the cells was substantially reduced for at least 24 hrs, and indeed they designated these cells as “exhausted NK cells” (Bhat and Watzl, 2007). Comprehensive in vitro investigations reported by Weiner’s group indicate that levels of CD16 are reduced considerably when NK cells mediate ADCC of RTX-

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opsonized cells, and these reductions in CD16 correlate with ADCC (Bowles and Weiner, 2005; Veermani *et al.*, 2011). In the absence of CD16 the NK cells cannot mediate ADCC and would clearly have an "exhausted" phenotype. Finally, Zent et al. recently reported that CD16 is also rapid reduced on circulating NK cells when CLL patients are treated with low doses of RTX, providing powerful in vivo evidence for reaction of NK cells with RTX-opsonized circulating CLL cells (Zent *et al.*, 2014). Presumably, due to the low dose treatment, levels of CD16 would be expected to return in a few days, but this issue has not yet been directly addressed.

Possible generalization to other immunotherapeutic mAbs

Obinutuzumab (OBZ; GA101) is a glyco-engineered Type II CD20 mAb that was recently approved for the treatment of CLL. A phase 3 study demonstrated substantial efficacy for OBZ plus chloroambucil in the treatment of CLL (Goede *et al.*, 2014). The cytotoxic mechanisms utilized by OBZ *in killing CLL cells* are still not completely defined, but considerable evidence indicates that, in common with RTX and OFA, effector functions likely play major roles in its cytotoxic action (Bologna *et al.*, 2011). We are unaware of any correlative studies for OBZ to date that are comparable to the studies we have cited for RTX and OFA, but other Type II CD20 mAbs are capable of promoting trogocytosis of CD20 in vitro (Pedersen *et al.*, 2011).

Many of our findings of trogocytosis and effector function exhaustion with respect to CD20 mAbs in CLL have been replicated and extended, in some cases to other mAb-

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antigen pairs, in the clinic and in the laboratory (Boross *et al.*, 2012; Jones *et al.*, 2012; Masuda *et al.*, 2013; Rossi *et al.*, 2013; Baig *et al.*, 2014). Therefore, the implications of these studies with respect to use of CD20 mAbs may also pertain to other mAbs currently used to treat cancer. Indeed, it was first thought that the principal mechanisms of action of both cetuximab and trastuzumab were based on direct cell killing via signaling and downstream apoptotic mechanisms. However, several recent reports, both in pre-clinical models and based on correlative studies, strongly suggest that a substantial component of their cytotoxic mechanisms is derived from cellular effector functions mediated by Fcγ receptors on monocytes, macrophages, and NK cells.

For example, trastuzumab had considerable efficacy in suppressing human tumor cell growth in a xenograft mouse model; however, the mAb had only modest activity in a comparable study in mice in which the common γ chain was knocked out, thus eliminating Fcγ receptor-mediated activity on effector cells (Clynes *et al.*, 2000). Fcγ receptor IIIA polymorphisms correlated with response to trastuzumab in breast cancer patients, a correlation that also has been reported for RTX therapy in patients with non-Hodgkin's lymphoma (Musolino *et al.*, 2008; Cartron *et al.*, 2002; Weng and Levy, 2003). Indeed, Varchetta et al. have found that CD16 is severely reduced on NK cells when they promote ADCC of trastuzumab-opsonized cells, suggesting that NK cell exhaustion may also be associated with trastuzumab therapy of breast cancer (Varchetta *et al.*, 2007). Finally, stimulation of CD137 on NK cells can increase ADCC of both trastuzumab-opsonized breast cancer cells as well as of RTX-opsonized B cells, again implying that cell-killing mechanisms mediated by Fcγ receptors on effector cells also obtain for trastuzumab (Kohrt *et al.*, 2011; Kohrt *et al.*, 2012).

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Evidence that cetuximab makes use of cell-based effector mechanisms to eliminate cancer cells derives from clinical correlative studies and in vitro investigations. Polymorphisms in Fcγ RIIA and Fcγ RIIIA correlate with increases in progression-free survival for colorectal cancer patients treated with either single agent cetuximab, or with cetuximab plus irinotecan (Zhang *et al.*, 2007; Taylor *et al.*, 2009; Botta *et al.*, 2012). CD20 is also decreased on targeted cells in tissues after RTX infusion (Laurent *et al.*, 2007; Teng *et al.*, 2007), but there is little evidence to indicate whether or not the analogous reaction occurs in vivo after infusion of cetuximab or trastuzumab. We have demonstrated that both cetuximab and trastuzumab can promote the shaving reaction in vitro (Beum *et al.*, 2008b). In view of the substantial tumor burdens associated with cancers that are being treated with these mAbs, it is likely that effector functions will be saturated in these cases as well.

Quantitative Considerations

The amount of mAb required to saturate the antigenic sites on a tumor can be far less than the quantities routinely administered. In the case of CLL, just 10 mg of an infused CD20 mAb will saturate 100,000 antigenic sites/cell on circulating CLL cells at levels of 100,000 cells per µl of blood (Lindorfer *et al.*, 2012)**.** More cells will re-equilibrate from other compartments, and the question of delivery to and penetration of liquid or solid tumors by the mAb constitutes an additional uncertainty (Jain and Baxter, 1988). These considerations provide reasonable justification for treating patients with large amounts of mAb. However, if the mAb requires effector functions to eliminate tumor cells, then treatment of a large tumor burden with a high mAb dose is likely to result in saturation of effector functions. The consequence, in the case of CD20 mAbs,

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is that opsonized cells are subject to trogocytosis; ironically, the excess CD20 mAb remaining in the circulation actually helps the circulating malignant B cells "escape" by promoting trogocytosis of mAb/CD20 complexes (Beum *et al.*, 2006). The pharmacokinetics and pharmacodynamics of RTX and of OFA have been comprehensively studied (Berinstein *et al.*, 1998; Coiffier *et al.*, 2010; Golay *et al.*, 2013b). At high doses RTX (and OFA) can persist in the circulation for several months, and we found that after infusions of large doses of these mAbs, CD20 levels on circulating CLL cells remained depressed over extended periods for up to one month or longer (Kennedy *et al.*, 2004; Beurskens *et al.*, 2012).

Based on our correlative observations in the clinical trials, in **Figure 2** we provide an idealized summary which compares and approximates the changes in the key measureable parameters that are evaluated under conditions of either high dose or low dose CD20 mAb therapy in CLL (Kennedy *et al.*, 2004; Beum *et al.*, 2004; Williams *et al.*, 2006; Beurskens *et al.*, 2012; Baig *et al.*, 2014; Zent *et al.*, 2014). There have been exceptions to these patterns, but the overall trends have been replicated in numerous studies. The key point (**Figure 2**) is that 48 hours after an injection of only 20 mg/m^2 mAb, CD20 is expressed at relatively high levels on CLL B cells (compared to pre-treatment) in the low-dose group, and in addition the body's immune effector functions have recovered and re-set from the first infusion, and thus are capable of clearing the next round of cells when more mAb is infused. A similar pattern for the low-dose group obtains for subsequent infusions at 48 hour intervals.

Concluding Remarks: The Way Forward

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On this basis we strongly suggest that analysis of the pharmacokinetics and pharmacodynamics of mAbs that *require immune effector functions* to eliminate cells may not provide the most important information with respect to efficacy and proper dosing, and that additional measurements should be made. In the case of CD20 mAb therapy in CLL, the level of CD20 on circulating B cells should be evaluated periodically to refine the treatment schedule. Additionally, we propose that dynamic monitoring of a patient's immune effector function status, including complement titer, and determination of the levels and fitness of circulating effector cells (expression of CD16 as well as of activation markers) to engage and kill mAb-opsonized cells (Bowles and Weiner, 2005; Berdeja *et al.*, 2007; Bhat and Watzl, 2007) will better inform the design and implementation of dosing paradigms.

We propose that a more reasonable and generally applicable dosing paradigm would be to periodically treat cancer patients with much smaller mAb doses, either iv at 30-40 mg, or subcutaneously at 50-60 mg to compensate for less efficient absorption (Golay *et al.*, 2013b) and to repeat these doses approximately three times per week (Williams *et al.*, 2006; Aue *et al.*, 2009; Zent *et al.*, 2014). The hypothesis is that each infusion will promote killing of a fraction of the tumor cells, and that trogocytosis will be minimized. Moreover, the effector systems will have time to recover based on this schedule, thereby allowing for a much higher degree of mAb efficacy. Recent evidence in support of the low dose paradigm for RTX was reported by Zent and colleagues, who examined the use of low but frequent doses of RTX in combination with pentostain and alemtuzumab in the treatment of progressive CLL (Zent *et al.*, 2014). They found that this approach constituted an effective therapy which was able to activate effector

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mechanisms without causing substantial loss of CD20. Moreover, Goldenberg's group has reported that lower doses of the CD20 mAb veltuzumab, given either iv or subcutaneously, also have demonstrable activity in the treatment of lymphoma (Morschhauser *et al.*, 2009; Negrea *et al.*, 2010). There is also additional, historic precedence for a low dose strategy. Alemtuzumab is a mAb specific for CD52 which is used in the treatment of CLL. This mAb also requires effector functions to promote CLL cell killing, and is given in either iv or subcutaneous doses of 30 mg, three times per week for extended periods (Zent *et al.*, 2004).

The treatment strategy we envision should be most effective if careful correlative measurements that monitor the patients' immune status are also conducted frequently, in effect allowing for more "personalized medicine" based on evaluation of laboratory parameters. Perhaps of most importance, if this approach were to prove successful and lead to equal or better outcomes compared to conventional high dose therapies, it would be relatively straight-forward to re-fashion this low dose paradigm into a subcutaneous injection strategy, which could make these treatments far more accessible and possibly more efficacious for patients.

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Acknowledgments

We thank the CLL patients who volunteered to participate in the correlative studies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Taylor, Lindorfer

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Molecular Pharmacology Fast Forward. Published on June 18, 2014 as DOI: 10.1124/mol.114.092684
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The work described in this review was supported by The Commonwealth Foundation for Cancer Research, a University of Virginia NIH Cancer Support Grant [P30CA044579], Genmab, and Glaxo Smith Kline.

Molecular Pharmacology Fast Forward. Published on June 18, 2014 as DOI: 10.1124/mol.114.092684
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Figure Legends

Fig 1. Both phagocytosis and trogocytosis occur simultaneously (Dabeuf et al, 2010). 38C13-CD20$^+$ B cells opsonized with Al488-RTX and dyed with PKH-26 were co-cultured for 30 min with adhered J774 macrophages. After the 38C13-CD20$^+$ B cells were removed, the adherent J774 cells were stained with Al647 anti-mouse IgM and then analyzed by fluorescence microscopy for Al488 RTX (**A**), PKH26 (**B**), and for Al647 anti-mouse IgM (**C**). Based on the images, selected regions in which the B cells were still adhered or in which the B cells had been either phagocytosed or trogocytosed by the J774 cells are identified. Original magnification, 40X. **Copyright 2010. The American Association of Immunologists, Inc.**

Fig 2. This idealized schematic summarizes the expected differences in key correlative parameters over 48 hrs when CLL patients receive high doses (either 375 mg/m^2 (~700 mg) or 300 mg) or low doses (20 mg/m^2; ~ 35 mg) of CD20 mAbs. The plasma concentrations of the mAbs immediately after completion of the respective first doses are ~ 100; 34; or 4 µg/ml. The results for individual patients are highly variable but the overall pattern that is displayed is representative of the general trends that have been observed. All values are relative to pre-treatment levels. **Cell Count** (Absolute lymphocyte Counts): Although levels of circulating CLL cells drop dramatically during infusion of the first 30 mg of mAb, re-equilibration from other compartments results in return to near pre-treatment levels in a few days for both high and low doses. **CD20/cell**: The default mechanism, trogocytosis, takes over after effector functions are saturated or exhausted with high doses of mAb. **Effector functions**: Complement titer

is sharply reduced with high doses, but only slightly reduced with low doses. When high doses are administered, ADCC and phagocytosis are exhausted and/or rendered ineffective due to trogocytosis.

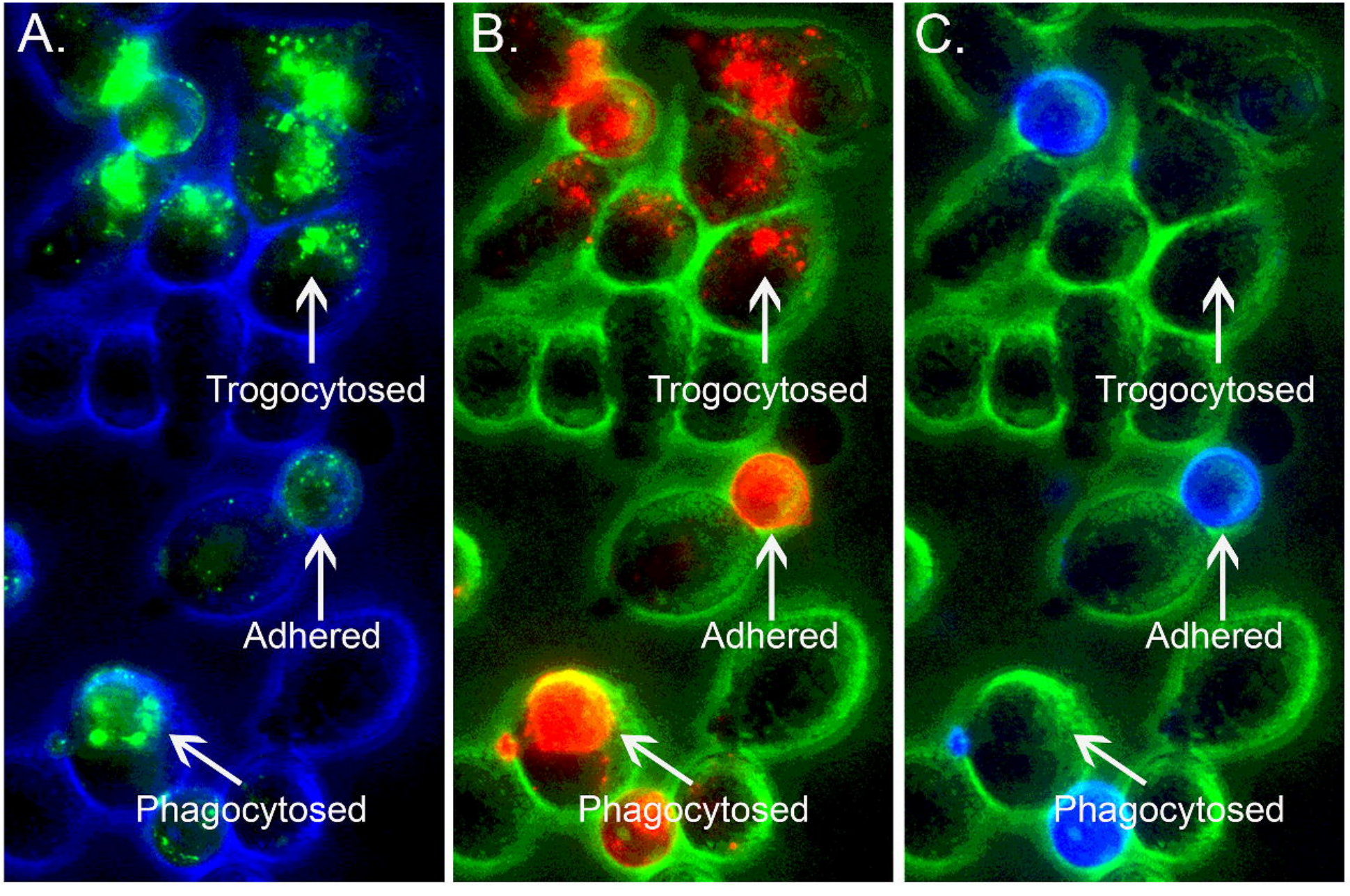
A.
Trogocytosed
Adhered
Phagocytosed
B.
Trogocytosed
Adhered
Phagocytosed
C.
Trogocytosed
Adhered
Phagocytosed

Figure 1

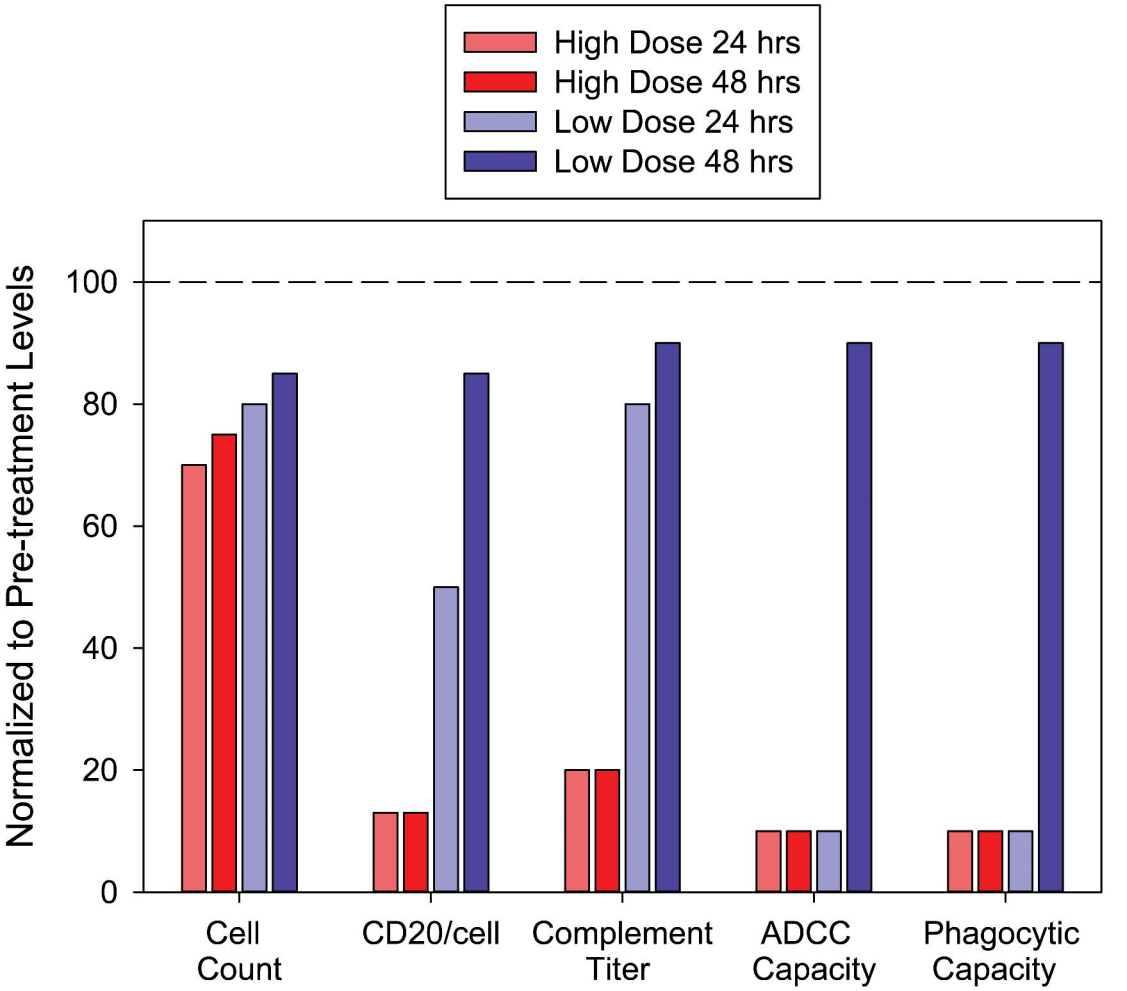
High Dose 24 hrs
High Dose 48 hrs
Low Dose 24 hrs
Low Dose 48 hrs
Normalized to Pre-treatment Levels
100
80
60
40
20
0
Cell Count
CD20/cell
Complement Titer
ADCC Capacity
Phagocytic Capacity

Figure 2