POTENTIATING ANTIBODY THERAPY BY TARGETING COMPLEMENT ON CANCER CELLS

by

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DEDICATION

I would like to dedicate this work to my parents, George and Cristie Carstens. Where would I be without your constant support? I have had so many great opportunities because of your hard work and love, I hope to never take it for granted and always do my best with the chance afforded me.

ABSTRACT

POTENTIATING IMMUNOTHERAPY BY TARGETING COMPLEMENT DEPOSITED ON CANCER CELLS

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Background:

Monoclonal antibodies (mAb) are a key component of treatment regimens for hematologic malignancies, but mAb-induced antigen loss on tumor cells can lead to treatment failure. Loss of cell surface CD20 can occur in the treatment of lymphoid malignancies with anti-CD20 antibodies (mAbs) (eg rituximab, ofatumumab) through trogocytosis. This is a frustrated form of phagocytosis, where the target CD20 antigen is removed with a piece of target cell plasma membrane by the immune effector cell, thus creating “escape variants”, which are no longer sensitive to the anti-CD20 therapy. In a clinical trial we initiated with anti-CD20 mAb ofatumumab in chronic lymphocytic leukemia (CLL), we observed that these escape variants carried covalently bound complement activation fragments, especially C3d. Indeed, C3d opsonized CLL cells persisted for weeks in circulation. (Beurskens et al., 2012). At final restaging after combined ofatumumab and

chemotherapy treatment, many patients had CD20 negative, but C3d positive disease. This suggests that a single mAb is insufficient to deplete cancer cells due to antigen escape.

Objective:

C3d may constitute a neoantigen that could be exploited to re-target cells that have escaped from anti-CD20 mAb therapy.

Methods:

To target complement opsonized cells we generated a human IgG1 mouse chimera mAb specific to C3d that is not competed by full length C3 in serum. To test whether targeting C3d can eliminate escape variants after anti-CD20 therapy, we collected blood samples from CLL patients before (Day 1) and 24 hours after administration of ofatumumab (Day2). To demonstrate that anti-C3d targeting was able to effectively circumvent antigen loss *in vitro*, we tested the anti-C3d mAbs ability to perform complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADCC), apoptosis and phagocytosis on Day 2 CLL cells. To test CDC, Day 2 CLL and previously treated CD20+ cell lines were incubated with anti-C3d mAb and normal human serum and stained for cell death. Similarly, ADCC was tested by coincubating Day 2 cells with an NK cell line in the presence of mAb. To evaluate phagocytosis, CLL cells were incubated with macrophages for six hours in the presence of antibody and imaged using flow cytometry to evaluate degree of internalization. We also tested the efficacy of the anti-C3d mAb *in vivo*, using two mouse models. First, we transferred peripheral blood mononuclear cells obtained

from CLL patients on Day 2 into NSG mice. Mice were treated with either isotype antibody, ofatumumab or anti-C3d mAb. Mice were sacrificed and tumor burden was quantified in peripheral blood and spleen. To evaluate impact on survival, we subcutaneously xenografted HBL2 cells, a CD20+ mantle cell lymphoma (MCL) line, into SCID mice. All mice received an injection of human C3 and either anti-CD20 mAb (rituximab or ofatumumab) alone, anti-CD20 and anti-C3d mAb or isotype control. Caliper measurements of the tumor longest dimension and survival were measured.

Results:

Anti-C3d mAb did not bind CLL cells obtained pre-treatment but bound cells obtained on Day 2. Day 2 CLL was effectively killed through CDC, NK cell mediated ADCC, and phagocytosis but not apoptosis. Phagocytosis of Day 2 CLL cells in response to anti-C3d treatment was two-fold higher than that observed on Day 1 CLL treated with ofatumumab. Importantly, non B lymphocytes were neither bound nor killed by the anti-C3d mAb, consistent with the highly targeted and selective deposition of C3d on CD20+ cells by ofatumumab *in vivo*. Our anti-C3d mAb effectively reduced tumor burden in both peripheral blood and spleen of the mice relative to mice treated with isotype control in the CLL primary NSG model. Our anti-C3d antibody extended time to tumor development and also prolonged survival in the MCL model differentially from CD20 targeting alone.

Conclusion:

Collectively, our results identify C3d as a marker for leukemic cells that have escaped *in vivo* antibody treatment and provide proof of principle evidence for the clinical utility of

C3d-targeting. In essence, we present an approach to utilize endogenous complement molecules as targets for immunotherapy. This approach relies on prior treatment with monoclonal antibodies that activate the complement cascade and thus covalently link complement component C3d to tumor cells. We conclude that anti-C3d mAbs can potentiate the anti-tumor activity of complement-fixing antibodies and eliminate antigen loss variants that survive after antibody therapy.

TABLE OF CONTENTS

CHAPTER ONE: AN INTRODUCTION …… ……2

CHAPTER TWO: EXPERIMENTAL PROCEDURES……..7

CHAPTER THREE: RESULTS ……12

CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATIONS ……..30

LIST OF FIGURES……..34

REFERENCES…….35

VITAE……..37

CHAPTER 1: INTRODUCTION

Since the initial development of antibody therapy in the 1980s, monoclonal antibodies (mAb) have grown into the largest segement in biopharmaceuticals (Ecker et al., 2015). In 2016, of 22 new drug approvals by the FDA, 8 were antibodies, which reflects a steady trend of growth (U.S. Food and Drug Administration, 2017). They are applicable in a wide range of diseases such as inflammatory disorders, infectious disease and cancer (Buss et al., 2012). A major application of therapeutic antibodies is in cancer. Multiple clinical trials have shown that a combination of chemotherapy with monoclonal antibodies is an effective strategy for treating cancer, however antibodies alone are not usually curative.

Antibody heavy and light chains contain variable and constant regions. Combing the light chain (V_L and C_L) with the variable segment and one of the constant segments of the heavy chains (V_H and C_H1), are together referred to as the fragment antigen binding domain (Fab), which generates the specificity of the antibody to its cognate antigen and allows therapeutic targeting. The main types of anti-cancer antibodies are chimeric or humanized IgG isotypes. The most commonly used IgG1 subclass is able to recruit immune effector mechanisms to kill tumor cells through complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC), and phagocytosis. Some antibodies are able to induce apoptosis of target cells or alter the immune milieu through direct interference with target cell signaling or the surrounding microenvironment (Scott et al., 2012).

Non-Hodgkins lymphoma (NHL) is the most common hematological cancer in adults, with approximately 72,580 new cases in 2016 (Siegel et al., 2016). NHL is a heterogenous group of diseases where about 85% originate from B cells and range clinically from

indolent and aggressive subtypes. Chronic lymphocytic leukemia (CLL), belongs to the group of NHL and is the most common leukemia in the Western world. CLL is characterized by a slow and progressive accumulation of abnormal mature B cells. Due to its slow growth, many patients may never become symptomatic from their CLL, however those with adverse cytogenetics may have a progression free survival of only 2 years even with standard treatment combination of chemotherapy and antibodies (chemoimmunotherapy), reflecting the innate heterogeneity of the NHLs (Wiestner, 2015).

Treatment with antibodies relies on targeting an antigen specific to the tumor. In hematological malignancies, this is most often a cell surface cluster of differentiation molecule (CD). CD20 is B cell specific antigen expressed on most NHL, but not on very early B cell progenitors, or their terminally differentiated plasma cell (Tedder et al., 1988). The protein is a membrane spanning calcium channel, which has no known ligand, but is responsible for efficient development and proliferation of the B cell population. The single most commonly used class of agents for combination therapy in B cell malignancies are anti-CD20 mAbs (Cheson and Leonard, 2008). While anti-CD20 can be used as a single agent in some indolent NHL, it has limited utility in most B-cell malignancies, and is consequently combined with chemotherapy. However, partly due to their specific and orthogonal mechanism of action relative to conventional chemotherapy, anti-CD20 mAbs are nearly universally combinable with different chemotherapies with minimal added toxicity. These various combinations have lead to improved NHL treatment. This therapeutic success is reflected in the market share of the human/mouse chimeric anti-CD20 IgG1 mAb, rituximab (Rituxan), the first anti-CD20 mAb approved by the FDA. In

2015, rituximab sales reached $7.1 billion, making it the top selling cancer drug (pharmaceutical-technology.com, 2016). Newer generation anti-CD20 antibodies, obintuzumab (Gazyva) and ofatumumab (Arzerra), have subsequently been developed and approved.

Despite the marked improvements in NHL survival with the addition of anti-CD20 targeting, many CLL patients relapse within years of chemoimmunotherapy. Though all the determinants of patient response to immunotherapy are still poorly understood, a growing body of evidence points to antigen loss as an important factor that limits immunotherapy efficacy Therapy-induced generation of antigen escape variants can occur through various mechanisms, including mutations, alternative splicing (Sotillo et al., 2015), epigenetic changes (Jacoby et al., 2016), as well as *in cis* (Vaughan et al., 2015) and *in trans* cellular uptake (Taylor and Lindorfer, 2015), known respectively as internalization and trogocytosis. Of the latter two mechanism, trogocytosis has been shown to affect a much wider range of antigens, including CD20, HER2, EGFR, CD5 (Beum et al., 2008), CD22 (Rossi et al., 2013) and CD25 (Zhang et al., 2014), and occur most rapidly (Beum et al., 2011). In fact, we previously described near complete losses of CD20 antigen from the surface of chronic lymphocytic leukemia (CLL) cells within hours of treatment with the anti-CD20 mAb ofatumumab (referred here as “OFA”) (Beurskens et al., 2012). Importantly, we observed that CLL cells that lost cell surface CD20 during OFA treatment are nevertheless marked with C3d, an irreversible imprint of complement activation, and carried covalently bound C3d complement fragments for weeks in circulation (Beurskens et al., 2012). Based on these data, we hypothesized that C3d is a neoantigen that could be exploited to re-target cells that have evaded anti-CD20 mAb

therapy.

C3d is the terminal cell surface bound form of C3, the most abundant complement protein and the central component of all complement activation pathways (Yang, 2013). Upon activation of the complement cascade, C3 is cleaved by C3 convertases and cell surface deposited in the form of C3b (Ricklin et al., 2010). C3b can deposit additional molecules of C3b and initiate the formation of the membrane attack complex (MAC) that results in lysis of target cells (Taylor and Lindorfer, 2014). Upon further proteolytic processing, C3b is converted to C3d and can no longer mediate C3 deposition or complement-mediated cell lysis, but still plays an important role in labeling target cells for elimination by phagocytes (Taylor and Lindorfer, 2014) and facilitating the initiation of adaptive immune responses (Dempsey et al., 1996; Haas et al., 2004; Hess et al., 2000; Toapanta and Ross, 2006). Importantly, C3d contains previously buried epitopes that are revealed through the proteolytic processing of C3 (Gros et al., 2008; Janssen et al., 2006; Janssen et al., 2005; Nishida et al., 2006)

We hypothesized that the C3d epitopes exposed through proteolysis represent neoantigens that can be utilized to complement the anti-tumor efficacy of mAb therapy. Our ongoing Phase II clinical trial investigating the anti-CD20 mAb ofatumumab against CLL (NCT01145209) provides an opportunity to test this hypothesis in a relevant clinical setting. Ofatumumab treatment of CLL thus represents a model system for antibody-induced antigen loss that can provided insights into the feasibility of the C3d-targeting approach. Using this model system as well as a chimeric anti-C3d IgG1 antibody generated within the lab (here after referred to “anti-C3d mAb”), we demonstrate that C3d can be targeted therapeutically and utilized to eliminate antigen loss variants

generated during mAb therapy.

CHAPTER 2: EXPERIMENTAL PROCEDURES

Patients

Primary peripheral blood mononuclear cell samples were obtained from patients enrolled in our ongoing, investigator-initiated phase II study of OFA plus chemotherapy (NCT01145209). The study was approved by the local ethics committee and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Briefly, only treatment naïve CLL or SLL patients were eligible and all patients were treated with OFA and fludarabine. Patients with either del(17p) or del(11q) mutations (Dohner et al., 1995) were also treated with cyclophosphamide. Patient derived cells were cultured in AIM V media (Life Technologies).

Flow cytometry

Flow cytometry was performed using murine mAbs specific for CD5, CD14, CD19, CD20, CD21, CD35, CD46, CD55, CD56, CD59 (BD Biosciences) on FACS Canto II and Fortessa LSR flow cytometers (BD Biosciences). Cell death was assessed using TO-PRO3 (Life Technologies) and measurements of complement proteins were done with a C4d-specific murine mAb (Quidel) and the 1H8 and 7C12 antibodies kindly provided by Prof. Ron Taylor of the University of Virginia. These antibodies were directly labeled using the AlexaFluor 488 antibody labeling kit (Life Technologies) or detected using FITC goat anti-mouse IgG secondary antibody (Sigma). Absolute cell counts were obtained using 5.0-5.9μm AccuCountBlank Particles (Spherotech) according to the manufacturer's instructions. MESF values were obtained using Quantum FITC-5 MESF beads (Bangs Laboratories). Data were analyzed using FlowJo (TreeStar) and Prism (GraphPad) software.

As described previously (Beurskens et al., 2012), to measure remaining CD20 expression on cells from patients treated with OFA and to ensure complete detection of all CD20 sites and avoid any “masking” by persistent OFA opsonization from in vivo treatment, we reopsonized all samples with unlabeled OFA and then stained with a labeled anti-human IgG1 secondary, thus identifying any residual OFA retained ex vivo and the sites left unbound initially from in vivo treatment.

Western blotting

Day 1 and 2 CLL cells were lysed in buffer containing 50mM Tris, 150mM NaCl, 1% Triton, 0.25% sodium deoxycholate, 1mM sodium vanadate, and 1 Complete® protease inhibitor cocktail tablet (Roche Diagnostics, Manheim, Germany). Equal amounts of total cell lysates were subjected to SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% dry milk in PBT (PBS, 0.1% Tween 20) for 2 hours before adding primary antibodies against CD20 (Cell signaling, Fremont, CA) or γ-tubulin (Sigma, St. Louis, MO).

Immunohistochemistry

Bone marrow trephine biopsies were fixed either in B-Plus fixative, embedded in paraffin and processed for morphological evaluation using standard procedures. Immunohistochemical studies with anti-CD20 and anti-CD79a antibodies (Ventana Medical System, Tucson, AZ, USA) were performed using immunoperoxidase staining procedures on an automated immunostainer (Ventana Medical System, Tucson, AZ, USA) according to the manufacturer's instructions. Images were obtained via digital microscopy using an Olympus BX-51 microscope (Olympus America, Center Valley, PA, USA) equipped with a DPlan 40/0·65 numeric aperture objective and captured using an

Olympus DP70 digital camera system. Image panels were prepared using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

Cell lines

NK92 cells were obtained from ATCC. NK92 cells that stably express an FcγRIIIA receptor with the 158V/V polymorphism (NK92-CD16V cells) that confers higher affinity binding were a kind gift from Prof. Louis Weiner of Georgetown University (Binyamin et al., 2008). THP1 cells were obtained from ATCC. NK92 and THP1 cells were cultured in RPMI 1640 media with 2mM GlutaMAX (Gibco). THP1 cell media contained 10% fetal bovine serum (HyClone), while NK92 media contained 10% heat inactivated normal human AB serum (Innovative Research) and 500U/ml recombinant human IL-2 (National Cancer Institute preclinical repository).

Complement-dependent cytotoxicity assays

CLL cells were incubated with OFA (commercial supply) or anti-C3d mouse-human chimeric antibody for 15 minutes and then in 50% normal human serum (Innovative Research) for two hours, stained with TOPRO3 (Life Technologies) and then analyzed by flow cytometry. Trastuzumab (commercial supply) was used as an isotype control, while heat inactivated serum served as a negative control. HBL2 cells were incubated as above, for one hour, first with OFA then washed and incubated again with anti-C3d mAb for one hour, and analyzed by flow cytometry.

Antibody-dependent cellular cytotoxicity assays

CLL cells were incubated with OFA (commercial supply) or anti-C3d mouse-human chimeric antibody for 15 minutes, co-incubated with NK92-CD16V for six hours, stained

with TOPRO3 (Life Technologies) and then subjected to flow cytometric analysis.

ImageStream analyses of antibody-dependent cellular phagocytosis

Monocyte-derived macrophages (MDMs) were obtained from normal donor elutriation samples (NIH Research Apheresis Clinic) and cultured for three days in RPMI media with 10% Pooled Normal Human AB Serum (Innovative) and 100ng/ml recombinant human macrophage colony stimulating factor (PeproTech). Day 1 and 2 CLL cells were stained with Violet Proliferation Dye 450 (BD Biosciences), opsonized with OFA or anti-C3d mouse-human chimeric antibody for 15 minutes and then co-incubated with MDMs for six hours. After this incubation, phagocytosis was detected as the absence of staining by an anti-CD19 PE-Cy5-conjugation antibody (BD Biosciences), and MDMs were labeled with anti-CD11b and anti-CD14 mAbs conjugated to PE (BD Biosciences). Samples were then run on an ImageStream X Mark II (Amnis) imaging flow cytometer. Data was collected using INSPIRE and analyzed using IDEAS software (Amnis).

Leukemia and lymphoma mouse xenograft models

NOD/scid/IL-2Rγ^{null} (NSG) mice (JAX strain 5557) and CBySmn.CB17-*Prkdc*^{scid}/J (JAX strain 1803) obtained from The Jackson Laboratory were housed and handled in accordance with the guidelines set by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, Bethesda, MD. All animal experiments were carried out on an approved animal protocol. *In vivo* anti-tumor activity of anti-C3d chimeric antibody against primary CLL cells was assessed in NSG mice xenografted with peripheral blood mononuclear cells obtained from CLL patients 24 hours after OFA infusion (50x10^6 cells). After leukemic cell engraftment on day 1, mice were treated with 10mg/kg isotype control or anti-C3d chimeric antibody on day 2 and sacrificed on day 5 or day 12. Leukemic cell

counts were quantified before treatment (day 2), after a single dose (day 3), and at endpoint (day 5). Disease burden in the peripheral blood and spleens of the mice was quantified as previously described (Vire et al., 2014).

To assess efficacy of the anti-C3d mAb against lymphoma, mice were injected with $5x10^6$ cells HBL2 cells subcutaneously. After engraftment on day 1, early treatment mice were injected with 10mg/kg isotype control or anti-CD20 mAb (OFA or RTX) alone or anti-CD20 mAb with anti-C3d chimeric antibody on day 3. Additionally, all mice received co-injection with 10mg/kg human C3 (Complement Technologies) at the time of mAb treatment to allow for opsonization with human C3d. Tumor development was monitored by caliper measurement. Mice were sacrificed when the tumor became ulcerated or reached 2cm in its longest dimension.

Statistical analyses

Samples were compared by paired or unpaired t-tests, as appropriate, if both samples passed a D'Agostino omnibus K^2 normality test and had standard deviations that were not statistically different (F-test). Otherwise, Mann-Whitney (unpaired) or a Wilcoxon matched-pairs signed-rank tests were used, as appropriate. Survival data were analyzed using log-rank test.

CHAPTER 3: RESULTS

Anti-CD20 mAb therapy of chronic lymphocytic leukemia results in the loss of CD20 expression with concomitant deposition of C3d on tumor cells

Twenty-nine patients with chronic lymphocytic leukemia sequentially enrolled onto a clinical trial of chemoimmunotherapy (**NCT01145209**) donated blood and bone marrow samples for translational studies. Treatment consisted of the anti-CD20 mAb ofatumumab combined with fludarabine-based chemotherapy. All patients were previously untreated, 16 (55 %) had advanced Rai stage, 10 (34%) had high-risk cytogenetic lesions and were treated with triple combination of ofatumumab, fludarabine and cyclophosphamide, while the remaining 17 (62%) received only ofatumumab and fludarabine (Figure 1A). Twenty-six (90%) patients completed at least three of the planned six cycles and achieved an objective response, complete response in 13 (45%) and partial response in 13 (45%).

The infusion of ofatumumab on day 1 resulted in a statistically significant reduction in the absolute lymphocyte count (ALC) of 27% (IQR 5-43; $p<0.0001$) with more substantial reductions achieved with the addition of chemotherapy starting on day 2 (Figure 1A-B). Repeat infusion of ofatumumab on day 8 again resulted in only moderate reductions in ALC (Figure 1A). Prior work indicates that exhaustion of effector mechanisms and loss of target antigen may limit activity of anti-CD20 mAb therapy (Beurskens et al., 2012). Indeed, when comparing tumor cells before (Day 1) and after (Day 2) exposure to ofatumumab *in vivo,* Day 2 cells showed near complete loss of CD20 protein expression by immunoblotting (Figure 1C) and flow cytometry (Figure 1D, median reduction 85.1% (IQR 80.9-89.9%, n=26). Although there was partial recovery of CD20 with time, CD20 levels never returned to baseline and significantly decreased after each subsequent

ofatumumab infusion (Figure 1E). Concurrently with the loss of CD20, CLL cells acquired C3d, a complement component covalently attached to cells reacted with a complement fixing mAb (Figure 1F) and remained C3d opsonized in between anti-CD20 antibody infusions (Figure 1G).

Twenty-six (90%) of 29 patients completed at least three of the planned six cycles and were evaluated for response. Responses by IWCLL criteria were complete response in 13 (50%) and partial response in 13 (50%). Eighteen (69%) of the 26 evaluable patients had flow cytometric evidence of residual disease at 10 months. The residual disease in the bone marrow after 3 cycles and completion of therapy had low or no CD20 expression as determined by immunohistochemistry for an intracellular CD20 epitope (Figure 1H) and flow cytometry (Figure 1I). In contrast, C3d was readily detectable on CLL cells at the interim as well as at the final restaging (Figure 1I). Based on these observations, we developed the concept that anti-C3d targeting could help eradicate cancer cells that dodge anti-CD20 mAb therapy.

Anti-C3d mAb induces complement-dependent cytotoxicity and amplifies target cell antigen density through additional C3d deposition

Having developed a chimeric mAb with strong, selective binding to C3d, we sought to determine whether anti-C3d mAb is selective for cells specifically targeted by the preceding complement fixing antibody. Both CD20 and CD19 are selectively expressed on B cells, including CLL cells. We therefore used CD19 to distinguish CLL from non-CLL cells. In concordance with the specificity of ofatumumab for CD20-expressing cells, the anti-C3d mAb only bound CD19+ CLL cells in the Day 2 samples, but not the CD19- cells and did not bind to any cells in Day 1 PBMCs (Figure 2A). In fact, Day 2 CD19+ cells

exhibited a 44-fold mean increase in C3d MESF values, compared to Day 1 cells (Figure 2B, $p<0.0001$). In contrast, there was no increase in anti-C3d binding to CD19- cells from ofatumumab treated patients. These results are consistent with the highly selective deposition of complement components onto ofatumumab bound cells and suggest that the targeting specificity of the anti-CD20 mAb is transferred to the anti-C3d mAb.

We next tested the ability of the anti-C3d chimeric mAb to mediate complement-dependent cytotoxicity (CDC). As expected, ofatumumab was able to mediate potent CDC against CLL cells obtained on Day 1, but was ineffective against CLL cells obtained after the patient received ofatumumab (Day2). In contrast, the anti-C3d mAb chimera left Day 1 CLL cells unharmed, but effectively induced CDC against Day 2 CLL cells. Notably, anti-C3d mAb-mediated CDC against Day 2 CLL cells was comparable to ofatumumab-mediated CDC against previously untreated CLL (Figure 2C). Anti-C3d mAb selectively induced CDC against Day 2 CD19+ cells, whereas Day 2 CD19- non-B lymphoctyes were not affected by ofatumumab or the anti-C3d mAb (Figure 2D). These results are in agreement with our observation that the anti-C3d mAb only binds Day 2 CLL cells, and not other cells from CLL patients treated with ofatumumab *in vivo*.

Anti-C3d mAb recruits NK-dependent cellular cytotoxicity

To assess the ability of the anti-C3d chimera to recruit NK cell cytotoxicity against C3d opsonized cells, we used NK92 cells that stably express FcyRIII (CD16). Ofatumumab was very effective at recruiting the NK92 cells against Day 1 CLL cells, but did not mediate significant antibody-dependent cellular cytotoxicity (ADCC) against Day 2 CLL cells (Figure 3D, $p<0.0001$, $n\geq8$ patients). Conversely, the anti-C3d mAb induced NK92 cells to kill Day 2 CLL cells, while leaving Day 1 CLL intact (Figure 2E). Notably, anti-C3d mAb-

induced lysis of Day 2 CLL cells was comparable to ofatumumab-induced lysis of Day 1 cells. Day 1 cells were not targeted by the anti-C3d mAb and Day 2 cells were not affected by ofatumumab, indicating that the lysis of CLL cells we observed is antibody-mediated (Figure 2E). Further, an anti-CD16 antibody blocked lysis by both therapeutic antibodies (Figure 2F), confirming that the observed killing is FcγR-dependent. The anti-C3d chimeric mAb did not directly induce apoptosis of CLL cells (Figure 2G), as expected for an antibody akin to a Type I anti-CD20 antibody that is capable of mediating CDC and ADCC, but not direct apoptosis (Beers et al., 2010; Bologna et al., 2011).

These data demonstrate the ability of anti-C3d mAbs to recruit potent complement and effector-cell mediated cytotoxicity against C3d-opsonized, CD20-antigen loss variants.

Macrophages recruited by anti-C3d mAb phagocytose C3d-opsonized leukemic cells

Next, we evaluated the ability of the anti-C3d mAb to induce phagocytosis of C3d-opsonized leukemic cells by macrophages. Day 1 and 2 CLL cells were stained with Violet Proliferation Dye (VPD, BD Biosciences), treated with ofatumumab or anti-C3d mAb *in vitro,* and co-incubated with monocyte-derived macrophages (MDMs) for six hours. Cells were then collected and stained. MDMs were identified with PE conjugated mAbs against both CD11b and CD14. Cells were analyzed by Imagestream and dual staining (VPD+ PE+) events containing both macrophages (PE+) and CLL cells (VPD+) were selected for further inspection (Figure 3A). Predictably, ofatumumab treatment resulted in a high number of dual positive or “interacting” events in Day 1, but not Day 2 samples. Inversely, with anti-C3d mAb treatment double positive events were frequent in Day 2 samples, but almost absent in Day 1 samples. Importantly, the anti-C3d mAb mediated a similar

number of co-stained VPD+ PE+ events in Day 2 CLL samples as ofatumumab did in Day 1 samples (Figure 3B).

To better understand the nature of the dual positive interactions we observed, we sought to determine what proportion of the dual positive events represented true internalization, rather than a superficial interaction. At the end of the coincubation the mixture of CLL cells and macrophages was stained with a PE-Cy5 conjugated anti-CD19 mAb differentiating events where CLL cells are in proximity to MDMs but not internalized, from events where CLL cells have been fully ingested by MDMs, and thus are no longer accessible to the CD19 mAb (Figure 3C). The VPD+ PE+ dual staining events were then divided into CD19+ events and CD19- events, the latter identifying CLL cells fully internalized by the macrophages (Figure 3D).

In aggregate, we found that exposing Day 2 CLL cells to anti-C3d mAb lead to a two-fold increase in internalization relative to naïve, Day 1 CLL exposed to ofatumumab. (Figure 3E-F). These data indicate that the anti-C3d mAb is equally effectively in recruiting phagocytes as ofatumumab but actually leads to more effective internalization of the tumor cell.

Anti-C3d mAb greatly reduces tumor burden in a CLL xenograft model

Based on our preliminary findings on the species specificity of the anti-C3d mAb, we decided to adapt the NOD SCID IL-2Rγ knockout (NSG) mouse model of CLL (NSG-CLL) to circumvent the lack of cross-reactivity with murine C3d. We injected NSG mice with Day 2 CLL cells, obtained from patients 24 hours after ofatumumab infusion, cells which have lost CD20 and been opsonized with human C3d. We then treated the mice with anti-C3d mAb or the negative control mAb trastuzumab on two different treatment schedules

and finally sacrificed the mice to collect peripheral blood and spleens (Figure 4A). The anti-C3d chimera demonstrated potent activity and decreased disease burden in the blood (Figure 4B-C) and spleens of xenografted mice (Figure 4D) treated with mAb the day after CLL cell injection. No adverse effects to mice or murine PBMCs were observed. A similar effect was observed in mice treated 4 and 6 days after CLL cell injection (Figure 5A -C).

Although we demonstrated effective CDC in vitro by the anti-C3d mAb, NOD mice have a deficiency in the complement component C5, therefore CDC is unlikely to play a significant role in the efficacy of the anti-C3d mAb in the NSG-CLL model. C5 is downstream of C3 in the complement cascade and thus does not affect labeling of target cells with the opsonins C3 and C4 (Baxter and Cooke, 1993). Notably, the anti-C3d chimera was able to deposit mouse complement *in vivo* (Figure 5D), which demonstrates that the anti-C3d mAb is capable of activating murine complement *in vivo*. This shows that CLL can be targeted effectively by attacking complement deposited on its surface as a result of prior mAb therapy, providing support for the potential *in vivo* therapeutic utility of C3d-targeting mAbs.

Anti-C3d mAb prolongs survival in a mantle cell lymphoma xenograft model

After observing promising activity of anti-C3d mAb in the NSG-CLL model, we set out to test the mAb in a model of mantle cell lymphoma (MCL). Like CLL, MCL is a B-cell malignancy that can be treated with anti-CD20 mAbs like ofatumumab. However, in contrast to the NSG-CLL model, MCL-SCID models can have fully functional cytolytic complement activity and are more appropriate for studying survival.

To provide the foundation for further in vivo experimentation, we assessed whether the

anti-C3d mAbs can mediate CDC against HBL2, a mantle cell lymphoma cell line. While CLL samples in our model were exposed to ofatumumab *in vivo*, and exposed to anti-C3d mAb ex vivo, we were able to see a similar level of CDC mediated against HBL2, a mantle cell lymphoma cell line, using sequential incubation with ofatumumab followed by anti-C3d mAb in the presence of normal human serum. In fact, following ofatumumab treatment with a C3d targeting mAb was even more successful in killing HBL2 cells than a second exposure to ofatumumab (Figure 6A). Notably, this increased killing occurred after only one hour and in the absence of monocytes, and thus without significant loss of CD20 from trogocytosis or internalization. This finding suggests that the anti-C3d mAb capable of improving upon the strong complement mediated cytotoxicity of ofatumumab, even in the absence of antigen loss.

After obtaining *in vitro* results demonstrating the susceptibility of HBL2 to anti-CD20 and anti-C3d combination, we assessed the ability of anti-CD20 and anti-C3d combination therapy to eliminate subcutaneously xenografted HBL2 cells in SCID mice. Mice received either ofatumumab or rituximab (anti-CD20) alone, anti-CD20 with anti-C3d mAb or isotype control (trastuzumab), either on day 3 or on days 14 and 21 after cell injection. All antibody treatments were accompanied by co-injection of human C3 (Figure 6B). While the CLL cells used in the NSG model were labeled with human C3d during patient treatment, HBL2 cells lack any C3 labeling at the time of xenografting. Supplying human C3 allows anti-CD20 treatment to deposit human complement onto the lymphoma cells in the SCID mouse, replicating *in vivo* opsonization in ofatumumab study patients.

Mice treated on day 3 with a combination of OFA or RTX with anti-C3d mAb showed longer time to tum or development than mice treated with isotype control or anti-CD20

mAb alone (Figure 6C). Even in mice treated on days 14 and 21, after gross tumor presentation, the combination of ofatumumab and anti-C3d showed improvements in time to tumor development (Figure 6D). The duration of survival of all mice organized by treatment group is shown in a Swimmer's plot (Figure 6E). On average, untreated mice succumbed by day 24, anti-CD20 treated mice lived as long as 38 days, while the combination treated mice lived 72 days, with 5 combination treated mice surviving until the study endpoint (Figure 6E). Collecting all treatment schedules according to treatment group, the combination of anti-CD20 mAbs with anti-C3d mAb more than doubled median survival, from 34 days to 79 days (Figure 6F).

Figure 1

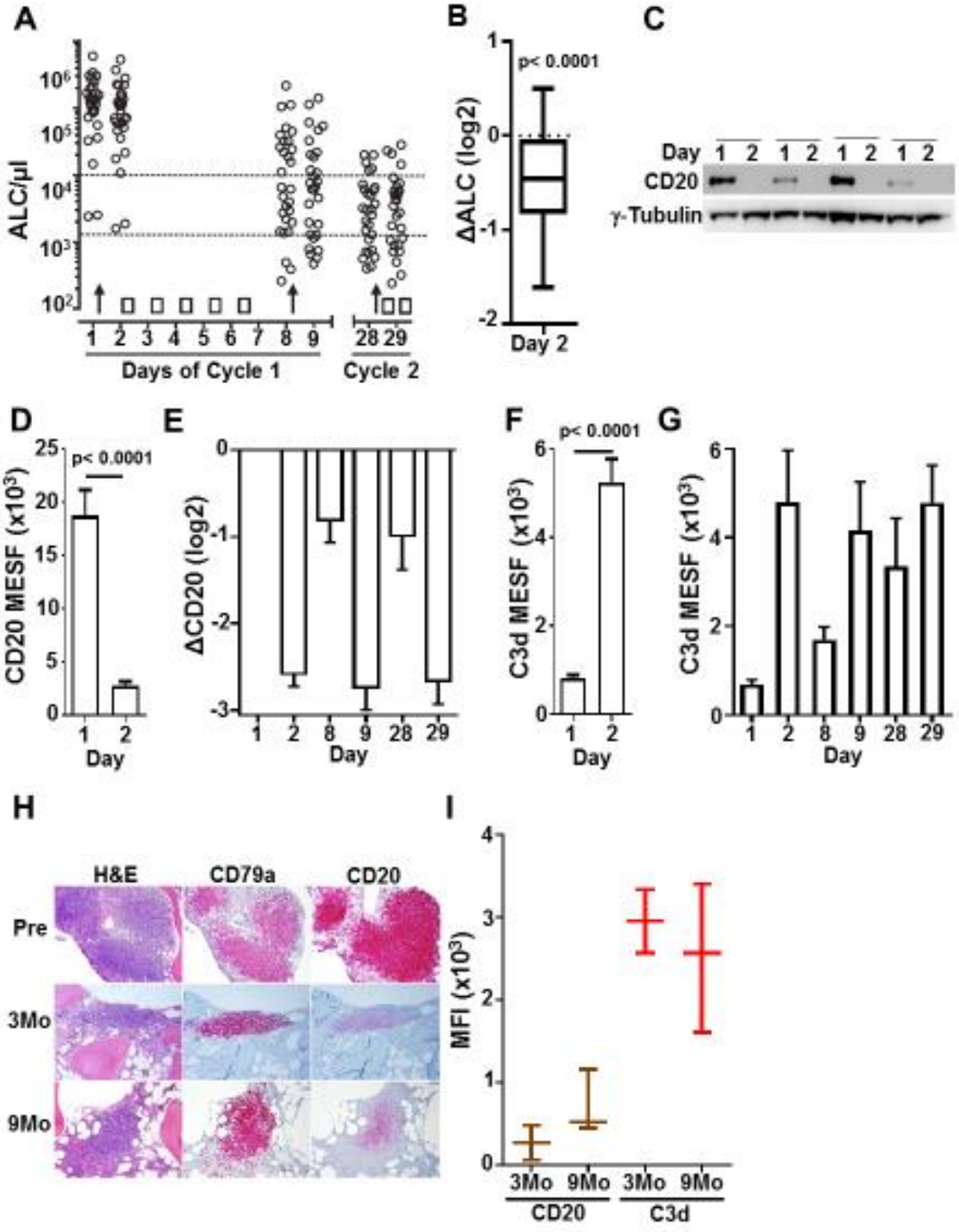

A
ALC/µl
Days of Cycle 1
Cycle 2
B
p< 0.0001
ΔALC (log2)
Day 2
C
Day
CD20
γ-Tubulin
D
p< 0.0001
CD20 MESF (x10³)
Day
E
ΔCD20 (log2)
Day
F
p< 0.0001
C3d MESF (x10³)
Day
G
C3d MESF (x10³)
Day
H
H&E
CD79a
CD20
Pre
3Mo
9Mo
I
MFI (x10³)
3Mo
9Mo
3Mo
9Mo
CD20
C3d

Figure 1. Anti-CD20 antibody therapy results in CD20 antigen loss and C3d deposition on leukemic cells.

(A) Absolute lymphocyte count (ALC) in 29 patients treated with the anti-CD20 mAb ofatumumab (arrows) on days 1 (300 mg), 8 (1 g) and 28 (1 g) and chemotherapy (open box) with fludarabine days 2-6 or the combination of fludarabine and cyclophosphamide days 2-4. Treatment was repeated on 28 day cycles for up to 6 cycles. Starting with cycle 2, ofatumumab (1g) was infused on the first day of each cycle. Blood samples were drawn immediately before and 24 hours after the start of each ofatumuamb infusion.

(B) Reduction in lymphocyte count within 24 hours of the first administration of ofatumumab (n=29, median 46%, IQR 7-80%).

(C) Western blot analysis of CD20 expression in CLL cell lysates obtained from 4 CLL patients before (Day 1) and after (Day 2) ofatumumab administration.

(D) Total CD20 before and after *in vivo* treatment with ofatumumab, stained *ex vivo* with saturating amounts of ofatumumab and anti-human IgG1 secondary antibody as described in Materials and Methods. n= 26

(E) Change of CD20 MESF after ofatumumab administration on days 1, 8, and 28 relative to pre-treatment baseline in 26 patients.

(F) C3d on CLL cells before (Day1) and after (Day2) ofatumumab administration. n = 26

(G) C3d MESF before (Day 1) and after ofatumumab administration relative to pre-treatment baseline. n= 26

(H) Bone marrow biopsies obtained pre-treatment (Pre), and after 3 (3Mo) and 9 (9Mo) months after initiation of treatment in a representative patient (OFA 23). Residual CLL is identified by staining for CD79a. Anti-CD20 antibody stain an intracellular epitope. Original magnification 100x

CD20 and C3d expression on bone marrow resident CLL cells assessed by flow cytometry in 3 patients; median and range is shown.

Figure 2

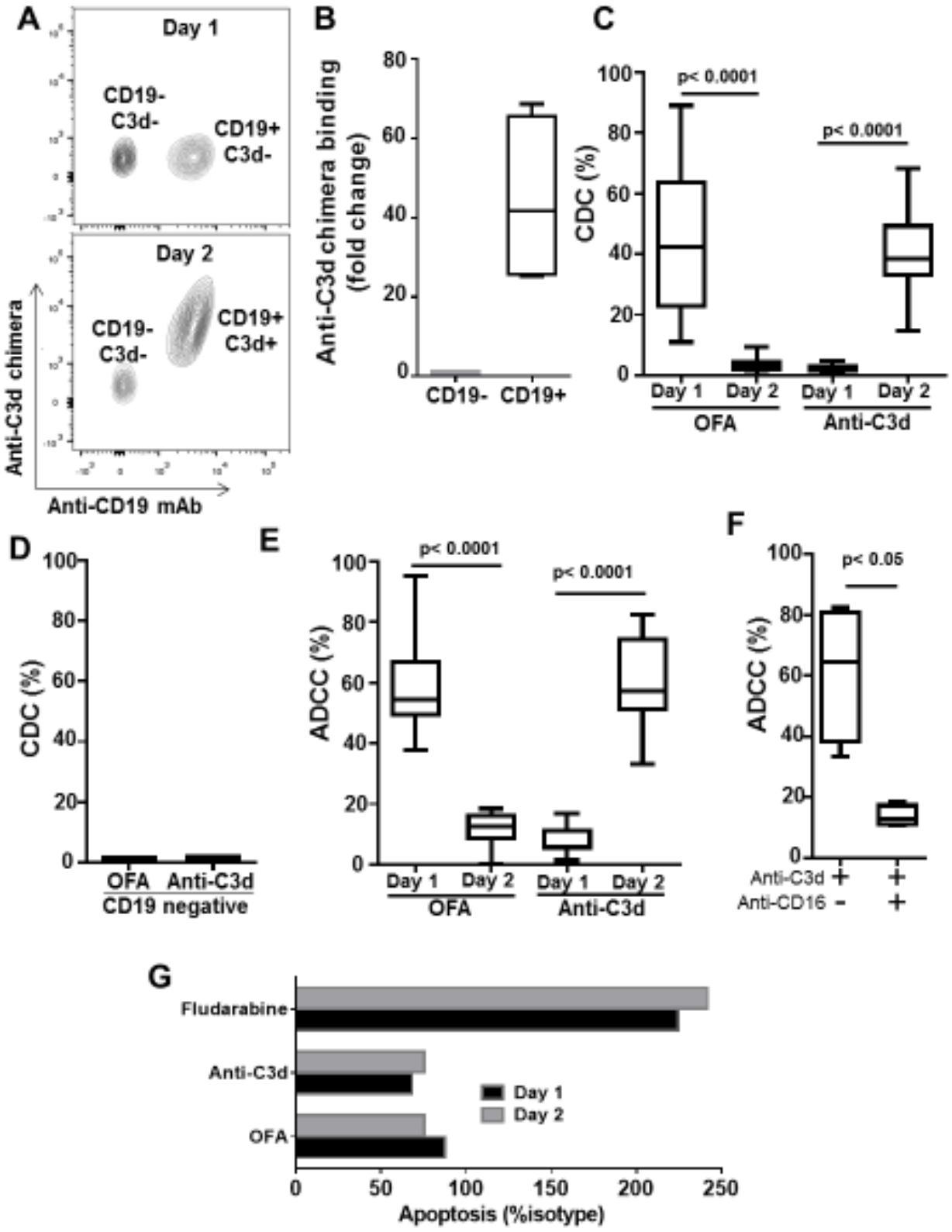

A
Day 1
CD19-
C3d-
CD19+
C3d-
Day 2
CD19-
C3d-
CD19+
C3d+
Anti-C3d chimera
Anti-CD19 mAb
B
Anti-C3d chimera binding
(fold change)
CD19- CD19+
C
CDC (%)
p< 0.0001
p< 0.0001
Day 1 Day 2 Day 1 Day 2
OFA
Anti-C3d
D
CDC (%)
OFA Anti-C3d
CD19 negative
E
ADCC (%)
p< 0.0001
p< 0.0001
Day 1 Day 2 Day 1 Day 2
OFA
Anti-C3d
F
ADCC (%)
p< 0.05
Anti-C3d + +
Anti-CD16 - +
G
Fludarabine
Anti-C3d
OFA
Day 1
Day 2
Apoptosis (%isotype)

Figure 2. Anti-C3d antibody selectively induces complement and cell-mediated cytotoxicity against C3d opsonized cells and amplifies C3d antigen density on target cells.

(A) PBMCs from a representative patient were stained with anti CD19 to identify CLL cells and anti-C3d chimeric mAb and analyzed by flow cytometry. Only CD19+ obtained after in vivo OFA treatment (Day2) are bound by anti-C3d mAb.

(B) Fold change in anti-C3d mAb binding to CD19- compared to CD19+ Day 2 PBMCs. Box and Whisker plots show median, IQR and range from 4 patients.

(C) Complement-dependent cytotoxicity (CDC) against CLL cells obtained from patients before (Day1) and after (Day2) OFA treatment mediated by OFA or the anti-C3d mAb measured in 18 patients via flow cytometry.

(D) CDC against CD19- cell from either Day 1 or Day2 mediated by OFA or the anti-C3d mAb. n = 18.

(E) Antibody dependent cellular cytotoxicity (ADCC) against Day 1 and Day 2 CLL cells mediated by either OFA or the anti-C3d mAb with NK92 cells. Mean ± SD from 8 patients shown.

(F) ADCC mediated by anti-C3d mAb on Day 2 cells with NK92 cells preexposed to either isotype or anti-CD16mAb. Mean ± SD from 4 patients shown

(G) Percentage of Annexin V+ (apoptotic) cells, normalized to isotype, performed on Day 1 and Day 2 CLL cells from n=1 patient, in duplicate.

Figure 3

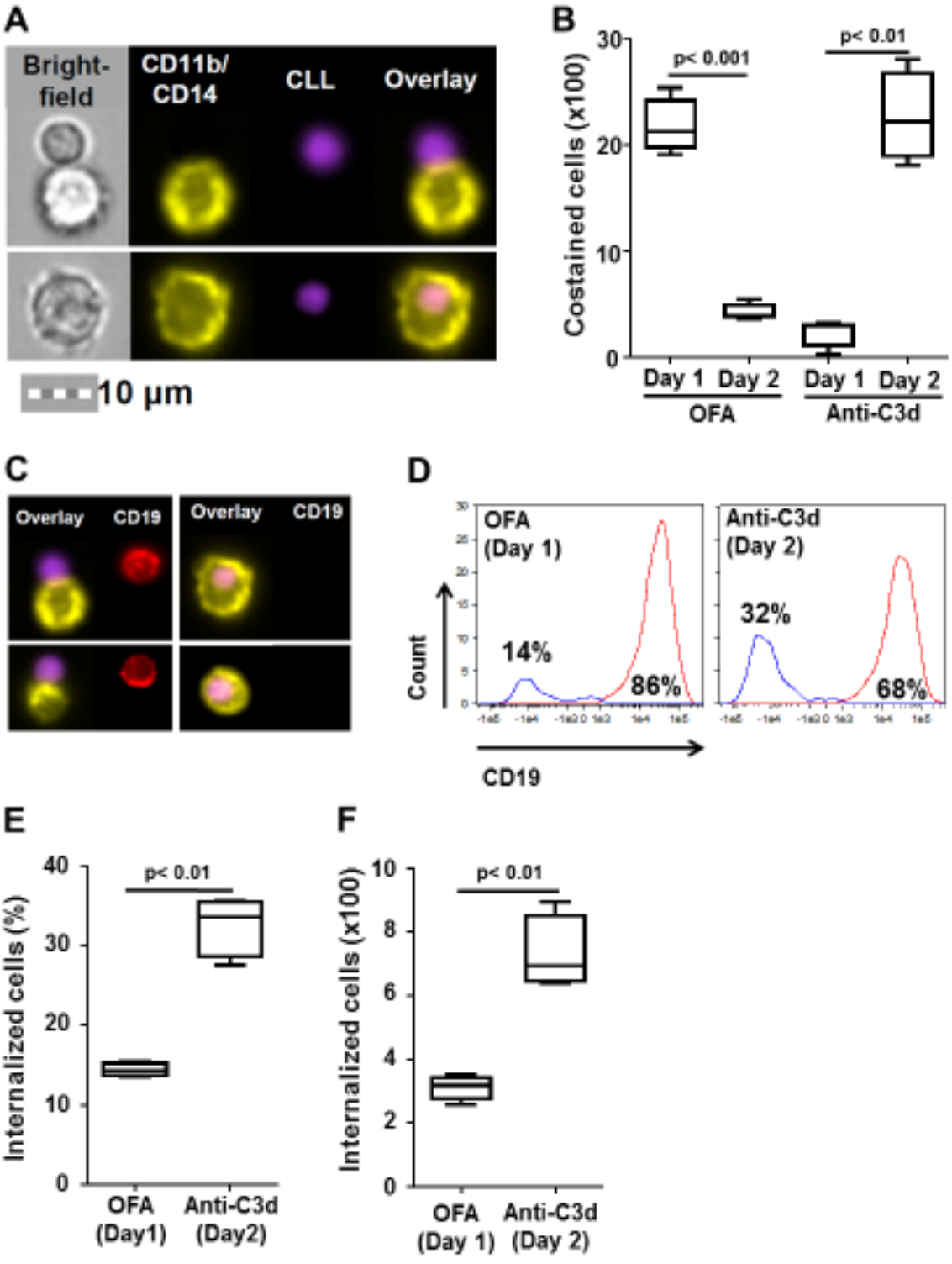

A
Bright-field
CD11b/ CD14
CLL
Overlay
10 µm
B
Costained cells (x100)
p< 0.001
p< 0.01
Day 1
Day 2
Day 1
Day 2
OFA
Anti-C3d
C
Overlay
CD19
Overlay
CD19
D
OFA (Day 1)
Anti-C3d (Day 2)
14%
86%
32%
68%
Count
CD19
E
Internalized cells (%)
p< 0.01
OFA (Day1)
Anti-C3d (Day2)
F
Internalized cells (x100)
p< 0.01
OFA (Day 1)
Anti-C3d (Day 2)

Figure 3: Anti-C3d mAb recruits cellular effector mechanisms against primary leukemic cells

(A) Representative micrographs showing MDMs in yellow, CLL in purple demonstrating “costained events”.
(B) The number of costained events obtained in four separate experiments in which PE-labeled monocyte derived macrophages were co-incubated with either ofatumumab or anti-C3d mAb and violet labeled Day 1 and Day 2 CLL cells. n = 4 patients
(C) Representative micrographs showing CLL cells (purple) and MDMs (yellow). Free CLL cells are detected with an anti-CD19 mAb (red) that was added after the 6 hour co-incubation of MDMs and CLL.
(D) Representative histograms showing the number of internalized CLL cells (red) and those merely interacting with or in the vicinity of MDMs (blue).
(E) The percentage of CLL which were fully internalized by MDMs. n=4 patients.
(F) Number of CLL cells in E.

Figure 4

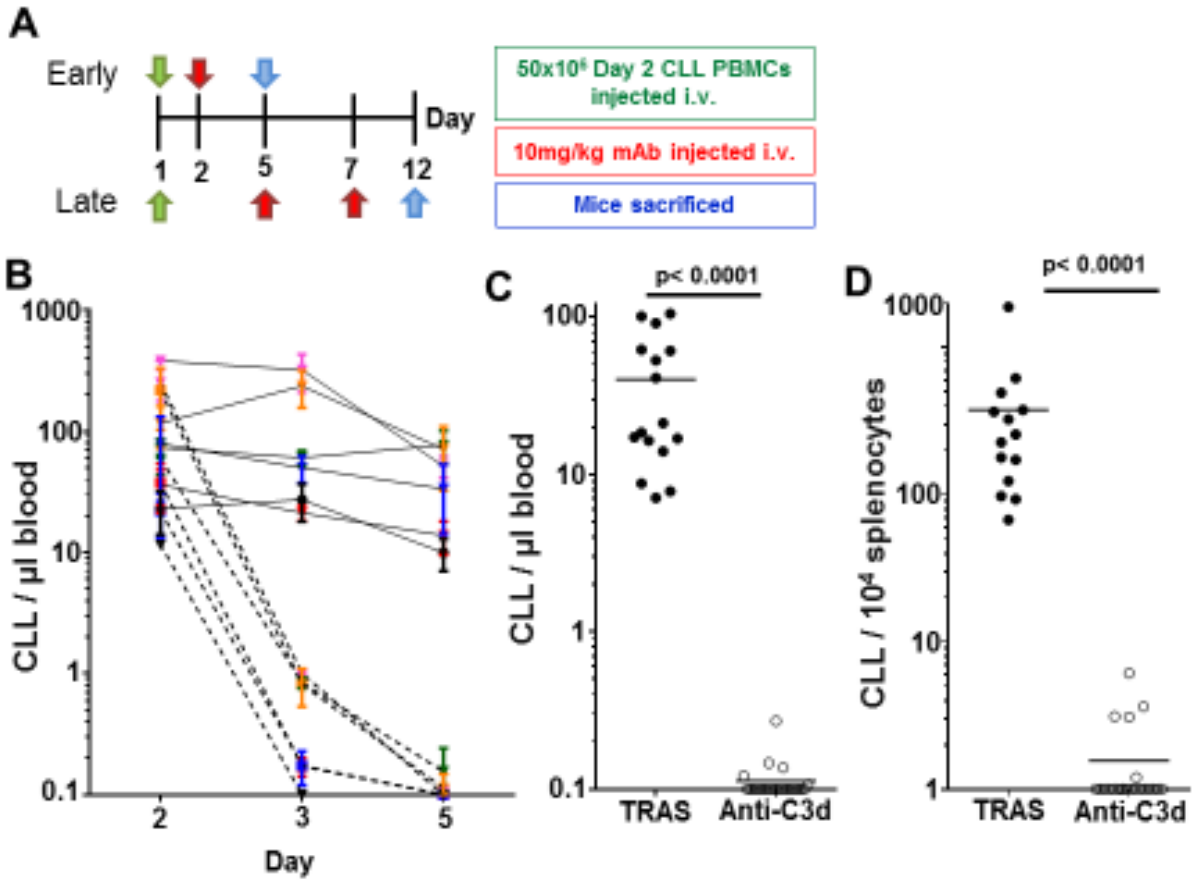


Figure 4. Anti-C3d mAb mediates potent anti-tumor activity in a xenograft model of chronic lymphocytic leukemia

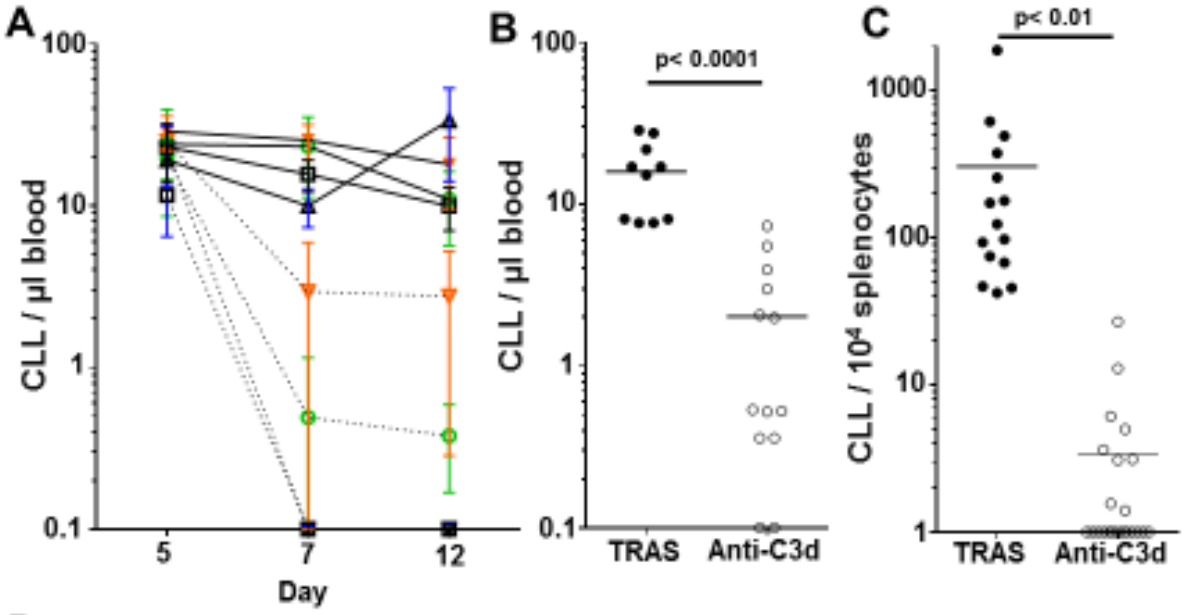
(A) Diagram showing experimental layout with early and late treatment schedules indicated on the top and bottom of day line respectively.

(B) Peripheral blood leukemic cell counts in NOD/scid/IL-2Rγ^{null} (NSG) mice xenografted (i.v.) with peripheral blood mononuclear cells from chronic lymphocytic leukemia (CLL) patients treated *in vivo* with OFA. Blood was obtained before treatment with 10mg/kg isotype control or anti-C3d chimeric antibody (day 2), one day after treatment (day 3) and before sacrifice (day 5). Solid lines denote isotype treated mice, while dashed lines indicate anti-C3d chimeric antibody treated mice. Colors represent different CLL patients (n=6) with 5-7 mice per patient.

(C) Leukemic disease burden in peripheral blood of NSG mice on day5 (n=34).

(D) Quantification of spleen resident leukemic cells on day5 (n=32).

Figure 5



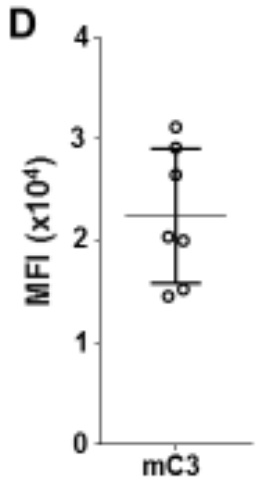


Figure 5. Anti-C3d mAb performs *in vivo* C3d opsonization with murine complement in a CLL xenograft model

(A) Average leukemic cell counts in peripheral blood that was obtained from NOD/scid/IL-2Rγ^{null} (NSG) mice xenografted with peripheral blood mononuclear cells from chronic lymphocytic leukemia (CLL) patients that were treated with ofatumumab *in vivo* (Day 2 CLL cells). After leukemic cell engraftment on day 1, mice were treated with 10mg/kg isotype control (solid line) or anti-C3d mAb (solid line) on days 5 and 7. Absolute quantification of leukemic cell numbers were performed before treatment (day 5), after a single dose (day 7) and before mice were sacrificed (day 12), (n=4 patients)

(B) Absolute counts of peripheral blood circulating leukemic cells in the peripheral blood of NSG mice on Day 12, (n≥10).

(C) Relative counts of and spleen resident CLL cells on Day 12, (n≥15).

(D) Mean fluorescence intensity (MFI) of anti-murine C3d on CLL cells obtained from peripheral blood on Day 12. The MFI of the isotype control is subtracted from all samples.

Figure 6

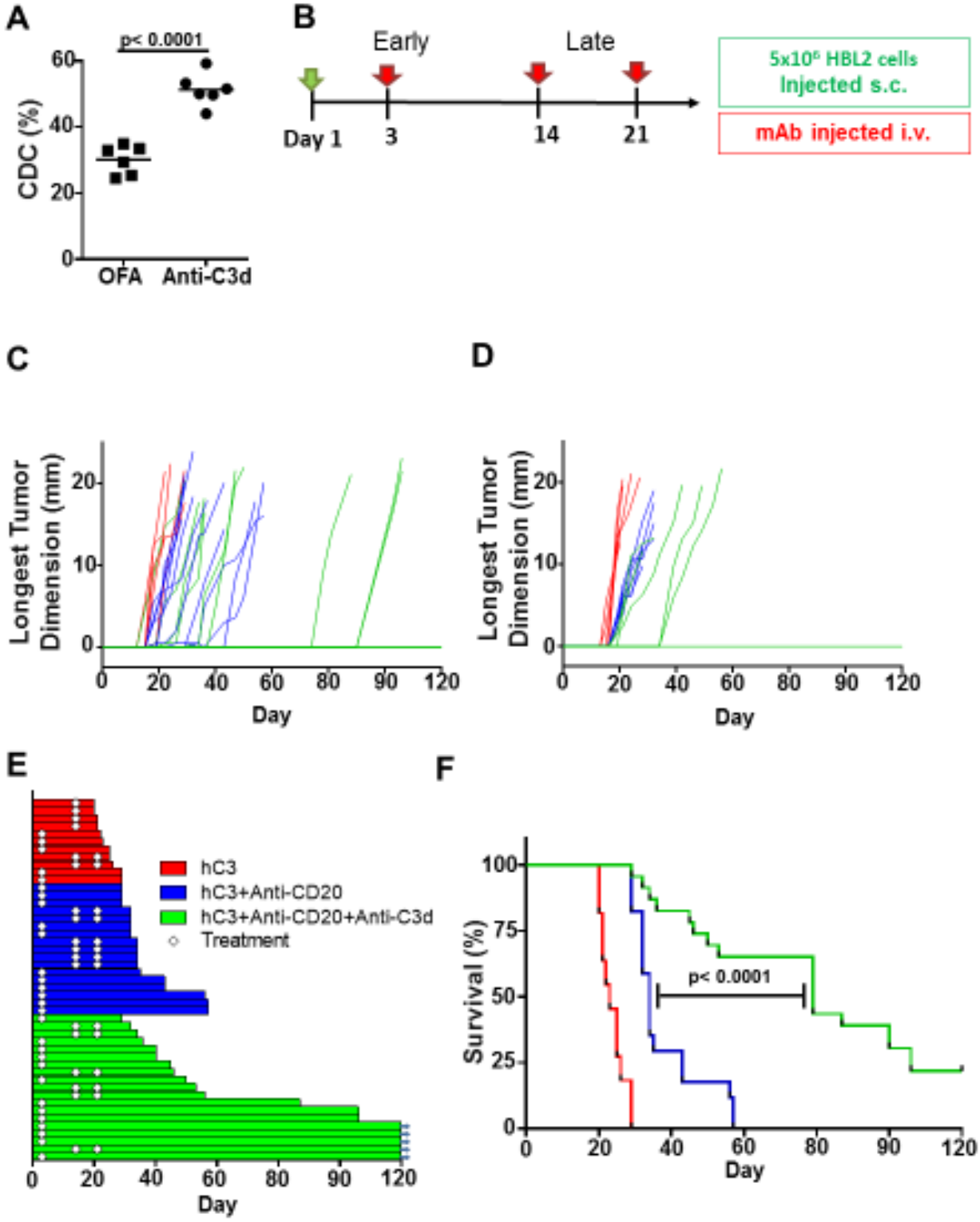

A
p< 0.0001
CDC (%)
60
40
20
0
OFA
Anti-C3d
B
Early
Late
Day 1
3
14
21
5x10⁶ HBL2 cells
Injected s.c.
mAb injected i.v.
C
Longest Tumor Dimension (mm)
20
10
0
0
20
40
60
80
90
120
Day
D
Longest Tumor Dimension (mm)
20
10
0
0
20
40
60
80
90
120
Day
E
hC3
hC3+Anti-CD20
hC3+Anti-CD20+Anti-C3d
Treatment
0
20
40
60
80
90
120
Day
F
Survival (%)
100
75
50
25
0
p< 0.0001
0
20
40
60
80
90
120
Day

Figure 6. Anti-C3d mAb mediates potent anti-tumor activity in a mantle cell lymphoma xenograft model

(A) CDC on HBL2 cells exposed to OFA and then Anti-C3d mAb in 25% NHS for 1 hour each, lysis is measured by TP3 positivity.

(B) Experimental layout showing early and late treatments, ongoing day line indicates mice were followed until tumor was ulcerated or reached 2cm in longest dimension, and then sacrificed.

(C) Caliper measurements performed on 29 SCID mice xenografted with HBL2 mantle cell lymphoma cells (s.c.) on day 1 were treated (i.v.) “early” on day 3 with human C3 and monoclonal antibodies. Caliper measurements similar to A, except human C3 and monoclonal antibodies were injected “late” on days 14 and 21, instead of day 3 (n=15).

(D) Swimmers plot of all mice. Any mice remaining alive at 120 days were euthanized. N=44

(E) Survival of mice described in B and C. P values obtained by log rank test compare 20 mg/kg Anti-HER2 alone (red), 20 mg/kg Anti-CD20 alone (blue) and 10mg/kg Anti-CD20 with 10mg/kg Anti-C3d (green). N=44

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

Here, we describe a novel approach to enhance the potency of mAb therapy in cancer. While cell-mediated, Fc-receptor dependent mechanisms appear to be critical for mAb-dependent cytotoxicity, most therapeutic mAbs used in hematologic malignancies also fix complement and deposit C3d on target cells. We engineered an anti-C3d antibody to test the hypothesis that targeting C3d could synergize with widely used therapeutic mAbs. In two *in vivo* models we demonstrate the power of this approach. First, using a patient-derived xenograft model, we observed that the anti-C3d mAb could effectively target antigen-escape variants arising in patients with CLL treated with anti-CD20 mAbs. Second, in an aggressive lymphoma model, the anti-C3d mAb synergized with anti-CD20 mAbs curing a subset of animals that, when treated with anti-CD20 mAbs alone, invariably succumbed to disease.

To target complement-opsonized cells, we generated a chimeric mouse/human IgG1 mAb against human C3d. As expected for a chimeric mouse/human IgG1 mAb, the anti-C3d mAb was able to kill target cells through CDC, NK cell mediated ADCC, and phagocytosis. Importantly, both binding to and killing of target cells was highly specific and limited to B cells previously bound by the anti-CD20 mAb. In particular, there was no binding to others cells, neither to T cells from patients treated with ofatumumab nor to non-B cells in PBMCs treated with anti-CD20 *in vitro*. In addition to these desirable but expected properties anti-C3d mAb demonstrated potentially very useful distinct mechanistic characteristics; retargeting of low antigen density cell types and highly effective phagocytosis.

Anti-C3d mAbs may be particularly valuable for targeting antigens with low surface density. For example, CD20 expression on CLL cells is generally lower than in other B-

cell malignancies (Naseem et al., 2015), and CD20 is prone to further downregulation by both internalization (Vaughan et al., 2015) and trogocytosis (Taylor and Lindorfer, 2015). Despite these limitations, CD20 expression is sufficient to allow for ofatumumab-mediated complement labeling of tumor cells. As we previously reported ofatumumab-mediated complement deposition can also occur in patients treated with the Bruton's Tyrosine Kinase (BTK) inhibitor ibrutinib which further decreased CD20 expression on CLL cells compared to pre-treatment levels (Skarzynski et al., 2016). These results indicate that antibody-mediated complement opsonization, in contrast to CDC, does not require high antigen density.

The nature of the target antigen imparts a second unique property on anti-C3d mAb. As a result of complement activation, C3d is covalently attached to a multitude of structures on the cell surface, including proteins, lipids, and glycans. This opsonization of target cells by complement is an integral part of innate immune responses and enhances ingestion of C3d coated cells by phagocytes through complement receptor 1. We observed that phagocytosis of anti-C3d coated CLL cells was more effective than phagocytosis of anti-CD20 coated cells (Figure 3). Notably, the difference appeared to arise not from a difference in the frequency of tumor-macrophage interactions but from a more efficient complete ingestion of anti-C3d opsonized cells by the phagocyte. We believe this may be due to a number of factors, including the ability of phagocytes to "hold" onto the target through both Fc-receptors and complement receptors and a decrease in the ability of target cells to escape by shedding parts of their membrane containing the antibody-antigen complex through trogocytosis.

We tested the efficacy of the anti-C3d mAb in two complementary mouse models. First,

in the NSG PDX we observed effective killing of CLL cells that had escaped ofatumumab based therapy in patients. Specifically, we transferred PMBCs obtained from CLL patients one day after administration of ofatumumab into NSG mice and treated these mice either with trastuzumab as a negative control or anti-C3d mAb. One injection of anti-C3d mAb reduced tumor burden in both peripheral blood and spleen by a median 99% (Figures 4) compared to trastuzumab treated mice. We conclude that anti-C3d targeting is effective against C3d opsonized tumor cells. In addition, these data support the concept that loss of CD20 antigen and not general apoptosis resistance is responsible for the persistence of tumor cells in CLL patients treated with anti-CD20 mAbs. A limitation of the PDX model is that transfer of CLL cells does not lead to death of the host precluding the use of survival endpoints (Durig et al., 2007).

In the second model of aggressive lymphoma, untreated mice died within 30 days and anti-CD20 antibody therapy extended survival, but all animals still succumbed to disease. In contrast, the combination of anti-C3d and anti-CD20 mAbs greatly extended the survival of the cohort likely curing a subset of mice that showed no evidence of disease for over 4 months, which is more than double the life-span of any anti-CD20 mAb treated animal (Figure 6E-F). Thus, anti-C3d synergized with anti-CD20mAb.

Our study focused on anti-CD20 antibodies and B-cell malignancies. This is the therapeutic arena in which antibody based cancer therapy originated with the development of rituximab. And to this day, rituximab remains the leading anti-cancer drug by sales. Nevertheless, we believe the approach described here can be applied beyond anti-CD20 to many therapeutic antibodies. Most anti-cancer antibodies are engineered using a human IgG1 backbone and will fix complement. Combination of anti-C3d with

therapeutic antibodies is therefore likely possible in different indications. For example, the recently approved first antibody targeting multiple myeloma, daratumumab, shares important characteristics with anti-CD20 mAbs, including strong complement deposition and the emergence of antigen-loss escape variants leading to treatment resistance.

In our present study, we demonstrate potent activity of a chimeric anti-C3d mAb with a wild type Fc region. Ongoing efforts to engineer improved antibody Fc regions may allow for the optimization of anti-C3d mAb-mediated complement deposition and CDC (Diebolder et al., 2014), and recruitment of FcγR-expressing immune effector cells (Kellner et al., 2014). Anti-C3d mAbs offer a versatile platform for engineering desired effector functions that through combination with existing therapeutic antibodies could be realized in different cancer types.

Collectively, our results provide proof of principle for the utility and potency of C3d-targeting. In essence, by harnessing complement opsonization of target cells by complement-fixing therapeutic antibodies, we present an approach to effectively deliver an “one-two-punch” attack on cancer cells.

LIST OF FIGURES

Figures 1: Anti-CD20 antibody therapy results in CD20 antigen loss and C3d deposition on leukemic cells.

Figure 2: Anti-C3d antibody selectively induces complement and cell-mediated cytotoxicity against C3d opsonized cells and amplifies C3d antigen density on target cells.

Figure 3: Anti-C3d mAb recruits cellular effector mechanisms against primary leukemic cells

Figure 4: Anti-C3d mAb mediates potent anti-tumor activity in a xenograft model of chronic lymphocytic leukemia

Figure 5: Anti-C3d mAb performs *in vivo* C3d opsonization with murine complement in a CLL xenograft model

Figure 6: Anti-C3d mAb mediates potent anti-tumor activity in a mantle cell lymphoma xenograft model

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VITAE

Elizabeth “Liz” Jane Carstens (April 30th 1989- present) was born in Shreveport, Louisiana to George and Cristie Carstens. She is the oldest of three, with a younger sister, Emily, and brother, John. She also has two wonderful step-parents Robin Carstens and Jack Reeves, and step siblings, Callea DeLong, Wade Bodgon, and Billy Reeves. She attended Holland Hall School in Tulsa, Oklahoma where she first developed a love of science, especially observational science, from her early field notes covering the changes of a square meter plot in the campus woods to later physics and biology labs (she still has the first agarose gel she ever ran). She completed her Bioengineering degree along with a minor in Global Health Technologies at Rice University in Houston, Texas in 2011. While there, she worked on a variety of designs targeted toward neonatal morality in the developing world, most notably a mechanical syringe pump based off a metronome. She also continued her research experience with Dr Junghae Suh, designing novel viral nanoparticles for cancer therapeutics. She will complete her MD at UT Southwestern in Dallas, Texas in June 2017. During her medical school training she also participated in the Medical Research Scholar Program at the National Institutes of Health in Bethesda, Maryland, where this work was performed under Dr Adrian Wiestner. She will be beginning her residency in Internal Medicine at Johns Hopkins in Baltimore, Maryland in July 2017.

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