The Addition of the BTK inhibitor Ibrutinib to Anti-CD19 Chimeric Antigen Receptor T Cells (CART19) Improves Responses against Mantle Cell Lymphoma

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ABSTRACT

Purpose: Responses to therapy with chimeric antigen receptor T cells recognizing CD19 (CART19, CTL019) may vary by histology. Mantle cell lymphoma (MCL) represents a B-cell malignancy that remains incurable despite novel therapies such as the BTK inhibitor ibrutinib, and where data from CTL019 therapy are scant. Using MCL as a model, we sought to build upon the outcomes from CTL019 and from ibrutinib therapy by combining these in a rational manner.

Experimental Design: MCL cell lines and primary MCL samples were combined with autologous or normal donor-derived anti-CD19 CAR T cells along with ibrutinib. The effect of the combination was studied in vitro and in mouse xenograft models.

Results: MCL cells strongly activated multiple CTL019 effector functions, and MCL killing by CTL019 was further enhanced in the presence of ibrutinib. In a xenograft MCL model, we showed superior disease control in the CTL019- as compared to ibrutinib-treated mice (median survival not reached versus 95 days, $p<0.005$) but most mice receiving CTL019 monotherapy eventually relapsed. Therefore, we added ibrutinib to CTL019 and showed that 80-100% of mice in the CTL019+ibrutinib arm and 0-20% of mice in the CTL019 arm, respectively, remained in long-term remission ($p<0.05$).

Conclusions: Combining CTL019 with ibrutinib represents a rational way to incorporate two of the most recent therapies in MCL. Our findings pave the way to a two-pronged therapeutic strategy in patients with MCL and other types of B-cell lymphoma.

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TRANSLATIONAL RELEVANCE

Most patients with relapsed mantle cell lymphoma can now be treated with the BTK inhibitor ibrutinib. However, up to 30% of these patients do not respond to ibrutinib and the majority of responders eventually relapse. Recent reports highlight potent activity of anti-CD19 chimeric antigen receptor T cells (CART19, CTL019) in B-cell malignancies. In this study we illustrate for the first time that ibrutinib can be added to CTL019 and that only the combined approach leads to profound, durable responses in xenograft models of MCL. These findings set the stage for future clinical trials evaluating this combination in B-cell neoplasms.

INTRODUCTION

Mantle cell lymphoma (MCL) accounts for up to 10% of all lymphomas (1) and typically presents in advanced stage. (2) For most patients with MCL the prognosis is poor with a median survival of four years. (3) Currently there is no curative treatment for MCL and, therefore, novel therapies for this type of lymphoma are urgently needed.

The B-cell receptor (BCR) complex is critical for antigen-induced activation of normal B lymphocytes and plays a key role in the pathogenesis of certain types of B-cell lymphoma. BCR engagement activates several kinases including LYN, SYK and BTK. (4, 5) BTK recently gained particular attention, since the potent BTK inhibitor ibrutinib demonstrated therapeutic efficacy in several types of B-cell lymphoma including MCL. (6-8) However, up to one third of MCL patients do not respond to ibrutinib and among the responders only a third achieve complete remission (CR). Furthermore, the therapy usually leads to drug resistance as the median duration of response is only 17.5 months with a 24 month PFS of 31%. (8, 9) The mechanisms of resistance are currently poorly understood but are thought to involve mutations in BTK that impair ibrutinib binding, or activating mutations of the enzyme PLCγ2 resulting in constitutive BTK-independent cell signaling. (10, 11) Furthermore, because blockade of BTK function is not directly cytotoxic, at least in some types of lymphoma, (11) it may predispose to clonal evolution by conferring a selection pressure. Rationally designed combinations of ibrutinib with other anti-lymphoma modalities could potentially overcome this shortcoming and thereby improve patient outcomes.

Infusion of autologous T cells transduced with chimeric antigen receptors (CAR) against the B-cell specific CD19 antigen (CART19, CTL019) leads to dramatic clinical

responses in many patients with various types of B-cell neoplasms but CTL019 efficacy against MCL specifically has not yet been established. (12-17) The presence of bulky masses may hinder T-cell infiltration with consequent impairment of anti-tumor activity. (18) Conversely, bulky lymphadenopathy does not appear to impair the response to ibrutinib and the drug actually triggers mobilization of the malignant cells to peripheral blood, potentially making them more accessible to CTL019 cells. (8)

In addition to BTK, ibrutinib irreversibly inhibits the TEC family kinase ITK (IL2-inducible T-cell kinase). ITK activates PLCγ upon T-cell receptor (TCR) ligation and leads to a signaling cascade that culminates in activation of T lymphocytes. (19) Recent preclinical data suggest that ibrutinib preferentially inhibits Th2-polarized CD4 T cells thus skewing T cells towards Th1 anti-tumor immune response. (20) However, another recent study shows that ibrutinib can antagonize rituximab-dependent NK cell-mediated cytotoxicity and reduce cytokine production, indicating that ITK inhibition may also lead to reduced tumor killing. (21) In this context, it is important to discover whether stimulation of the chimeric antigen receptor in CTL019 cells would lead to activation of ITK and if so, whether inhibition of ITK by ibrutinib would have an advantageous or deleterious effect on CTL019 function.

In principle, the combination of the BTK inhibitor ibrutinib with CTL019 brings together two leading novel approaches to the treatment of B-cell lymphoma and by taking advantage of their vastly different mechanisms of action may prove particularly effective. Using *in vitro* and *in vivo* models of MCL, including a novel cell line highly sensitive to ibrutinib, we demonstrate here that CTL019 is more effective than ibrutinib as monotherapy, and that the addition of ibrutinib to CTL019 further augments the anti-tumor effect and leads to prolonged remissions.

MATERIALS and METHODS

Cell lines and primary samples. Cell lines were originally obtained from ATCC (Manassas, VA) (K-562, Mino and JEKO-1) or DSMZ (Braunschweig, Germany) (MOLM-14 and NALM-6) (cell lines were obtained more than 6 months prior experiments and authentication was performed by cell banks utilizing Short Tandem Repeat (STR) profiling) while MCL-RL was generated in our laboratory from a pleural effusion of a MCL patient (the presence of the t(11;14) characteristic of MCL was tested by FISH). All cell lines were tested for the presence of mycoplasma contamination (MycoAlert™ Mycoplasma Detection Kit, LT07-318, Lonza, Basel, Switzerland). For some experiments, MCL-RL and JEKO-1 cells were transduced with firefly luciferase/eGFP and then sorted to obtain a >99% positive population. Cell lines MOLM-14, K562, and NALM-6 were used as controls as indicated in the relevant figures. The cell lines were maintained in culture with RPMI media 1640 (Gibco, 11875-085, LifeTechnologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gemini, 100-106, West Sacramento, CA), and 50 UI/ml penicillin/streptomycin (Gibco, LifeTechnologies, 15070-063). De-identified primary human MCL bone marrow (BM) and peripheral blood (PB) specimens were obtained from the clinical practices of University of Pennsylvania under an Institutional Review Board (IRB)-approved protocol (UPCC #03409). For all functional studies, primary cells were thawed at least 12 hours before experiment and rested at 37°C.

Fluorescence in situ hybridization (FISH) and immunohistochemistry. The FISH analysis and immunohistochemistry were performed according to the standard method and as previously described. (22) Specifics of the experiment of this paper are detailed in the **Supplementary Methods** section.

Immunohistochemistry. Thin-layer cell preparation was obtained by Cytospin (Thermo Scientific) and stained with Giemsa. For formalin fixed paraffin embedded tissues immuno-histochemical (IHC) staining was performed on a Leica Bond-III instrument (Leica Biosystems, Buffalo Grove, IL, USA) using the Bond Polymer Refine Detection System. Antibodies against CD2, SOX-11, Pax5 and CyclinD1 were used undiluted. Heat-induced epitope retrieval was done for 20 minutes with ER2 solution (Leica Microsystems, AR9640). Images were digitally acquired using the Aperio ScanScope™ (Leica Biosystems).

Generation of CAR constructs and CAR T cells. The murine anti-CD19 chimeric – antigen receptor (CD8 hinge, 4-1BB co-stmulatory domain and CD3 zeta signaling domain) was generated as previously described. (23) **(Fig S3 A)** Production of CAR-expressing T cells was performed as previously described. (24) **(Fig S3 B)**

Ibrutinib. Ibrutinib (PCI-32765) was purchased from MedKoo (#202171) or Selleck Biochemicals (#S2680) as a powder or DMSO solution. The products obtained from the two companies were compared and proven to have equivalent activity (data not shown). For in vitro experiments ibrutinib was dissolved in DMSO and diluted to 2, 10, 100 or 1000 nM in culture media. For in vivo experiment ibrutinib powder was dissolved in a 10% HP-beta-cyclodextrin solution (1.6 mg/ml) and administered to mice in the drinking water.

Multiparametric flow cytometry. Flow cytometry was performed as previously described (24, 25) and detailed characteristics of the experiments are provided in **Supplementary Methods**.

MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) enzymatic conversion assay. The assay was performed as previously described. (26) Specifics of the experiment of this paper are detailed in the **Supplementary Methods** section.

DNA fragmentation (tunel) assay. ApoAlert DNA fragmentation assay kit (Clontech, 630108, Mountain View, CA) was used according to the manufacturer's protocol. In brief, cells were cultured at 0.5 × 106 cells/mL for 72 h with DMSO (control) or ibrutinib at the listed doses. The cells were then washed, fixed, permeabilized, and incubated for 1 h at 37°C with or without TdT. After exposure to the stopping buffer and washing, the cells were analyzed by flow cytometry using the CellQuest PRO software v. 5 (BD biosciencies).

Western blot analysis. The assay was performed as previously described. (27) Specifics of the experiment of this paper are detailed in the Supplementary Methods section.

Real-time-PCR. CTL019 cells were screened by RT-PCR analysis for Fas ligand (AB, LifeTechnologies, Hs00181225_m1), granzyme B (AB, Hs01554355), perforin (AB, Hs00169473_m1) and TRAIL (AB, Hs00921974 mRNA expression at the end of expansion (day 10). RNA was extracted with RNAqueos-4PCR Kit (Ambion, LifeTechnologies, AM-1914) and cDNA was synthesized with iScript Reverse Transcription Supermix for RT-qPCR (BioRad, 170-8841). The relative target cDNA copies were quantified by relative qPCR (qPCR) with ABI TaqMan specific primers and probe set; TaqMan GUSB primers (AB, Hs00939627) and probe set were used for normalization.

In vitro T-cell effector function assays. CD107a degranulation, CFSE proliferation, cytotoxicity assays and cytokine measurements were performed as previously described. (24, 28) Specifics of the experiment of this paper are detailed in the **Supplementary Methods** section.

Animal experiments. In vivo experiments were performed as previously described. (24, 25, 29) Schemas of the utilized xenograft models are discussed in detailed in the relevant figures, result and **Supplementary Material** section.

Statistical Analysis. All statistics were performed as indicated using GraphPad Prism 6 for Windows, version 6.04 (La Jolla, CA). Student's t-test was used to compare two groups; in analysis where multiple groups were compared, one-way analysis of variance (ANOVA) was performed with Holm-Sida correction for multiple comparisons. When multiple groups at multiple time points/ratios were compared, the Student's t-test or ANOVA for each time points/ratios was used. Survival curves were compared using the log-rank test. In the figures asterisks are used to represent p-values (*=<0.05, **=<0.01, ***=<0.001, ****=<0.0001) and "ns" means "not significant" (p>0.05). Further details of the statistics for each experiment are listed in figure legends.

RESULTS

Sensitivity of MCL cell lines to ibrutinib

Most MCL cell lines in existence have been immortalized and propagated for many generations in vitro and are poorly sensitive to ibrutinib. (30, 31) We harvested MCL cells from the pleural effusion of a patient with an advanced MCL and established a cell line (MCL-RL) that retained the primary-cell polymorphic morphology, the characteristic MCL immunophenotype including CD19 and CD5 co-expression, and the classical t(11;14) translocation with 6-7 copies per cell of the IgH-Cyclin D1 fusion gene cell (**Fig 1A**). Exposure of the MCL-RL cell line to increasing concentrations of ibrutinib led to a dose-dependent inhibition of cell growth with an IC50 of 10nM (**Fig 1B**), including their apoptotic cell death (**Fig S1A, top row**). In contrast, the commonly-used MCL cell lines Mino and JEKO-1 were relatively resistant to ibrutinib, with IC50 of 1 μM and 10μM, respectively (**Fig 1B**) and showed no evidence of cell death (**Fig S1A, bottom row**). Of note, ibrutinib inhibited phosphorylation of BTK to a similar degree in both sensitive (MCL-RL) and resistant (JEKO-1) cell lines, indicating that the resistance in JEKO-1 cells is BTK-independent (**Fig S1B**). Non-MCL cell lines NALM-6 (B-cell acute lymphoid leukemia) and K562 (acute myeloid leukemia) were also tested for ibrutinib sensitivity showing IC50 of >1 and >10 μM, respectively (**Fig S1C**) and hence served as additional negative controls throughout the study. To determine suitability of MCL-RL cells for in vivo experiments, we injected immunodeficient NSG mice intravenously with $1x10^6$ MCL-RL cells expressing firefly luciferase and monitored the mice for tumor burden by bioluminescence imaging and for survival. The MCL-RL cells engrafted in all mice and localized predominantly to the spleen and liver, followed by dissemination to bone marrow, blood and other organs (**Fig S2A**). Histology and immunohistochemistry

of the tumors recapitulated the morphology and immunophenotype of the original MCL-RL cells (**Fig S2B**). Importantly, MCL-RL demonstrated response to ibrutinib treatment also in this *in vivo* setting with a dose-dependent reduction in tumor growth **(Fig. 1C, top panel)** and improvement in overall survival **(Fig. 1C, bottom panel)**.

Mantle cell lymphoma cells are sensitive to CTL019 effector functions

To examine sensitivity of the MCL cells to killing by CTL019 cells, we transduced healthy donor T cells with the same anti-CD19 CAR construct that has been used in our group's clinical trials (16) and used for the following experiments. The design of this CAR and the T-cell production schema are shown in **Fig S3A and B**. In order to test whether CTL019 cells could be manufactured also from the blood of patients with leukemic MCL, we expanded and transduced patient-derived T cells **(Fig 2A top panels)** then performed a CD107a degranulation and cytokine production assay to demonstrate reactivity against that patient's own MCL **(Fig 2 A bottom panel)**. Given the recent interest (32) in tumor-infiltrating and marrow-infiltrating lymphocytes, we also performed a similar study using marrow-derived T cells from a patient with stage IV MCL (**Fig 2A and Fig S4A and B**). A series of in vitro experiments showed that both the ibrutinib-sensitive MCL-RL and the ibrutinib-resistant JEKO-1 cell line induced comparably strong activation of CTL019 cells as determined by their degranulation, cytokine production, cytotoxic activity, and proliferation (**Fig 2B, C and Fig S4C**). As shown in Figure 2C the MCL-RL cell line was less sensitive to CTL019 cytotoxicity as compared to JEKO-1, This was likely due to increased activation-induced apoptosis of CTL in the presence of MCL-RL (**Fig S4D).** The CTL019 activation was strictly CAR-

dependent, since the untransduced cells (UTD) from the same donors tested in parallel showed no, or very limited, activity in these assays. We next evaluated in vivo different doses of CTL019 cells and demonstrated a dose-dependent anti-tumor efficacy, with $2x10^6$ CTL019 cells per mouse proving to be the most effective. Higher doses of T cells were associated with non-specific alloreactivity (data not shown). Notably, the anti-lymphoma activity of CTL019 was observed in NSG mice engrafted with both ibrutinib-resistant (JEKO-1) **(Fig 2D, top panel)** and ibrutinib-sensitive (MCL-RL) MCL cell lines **(Fig 2D, bottom panel)**. These results indicate that MCL is sensitive to the effector functions of CTL019 cells.

Impact of ibrutinib on CTL019 function in vitro

Ibrutinib was originally thought not to impact T cells based on short-term activity assays. (33) However, a comprehensive analysis of the impact of ibrutinib on the T cell kinase ITK subsequently supported an overall immunomodulatory role of ibrutinib in CD4 T cells as suppressor of Th2-type polarization. (20) Cytokine expression pattern analysis of patients treated with anti-CD19 CAR T cells performed by several groups indicates that this therapy is associated with both Th1-type (IL2, IFNγ, TNF) and Th2-type (IL-4, IL-5, IL-10), as well as other cytokine-secretion patterns. (13, 28) Therefore, we evaluated the effect of ibrutinib on CTL019 function at, above, and below the concentrations that would be expected in patients (mean peak concentration in patient serum is 100-150ng/ml). (6) We found that CTL019 cells express ITK and that stimulation of CTL019 cells, whether through the TCR complex or through the CAR, led to phosphorylation of ITK. The presence of ibrutinib resulted in a modest reduction in

ITK phosphorylation that was only evident at the highest concentration of ibrutinib (**Fig 3A**).

We next probed the short- and long-term *in vitro* function of CTL019 cells in the presence of ibrutinib. Following 4-6 hours of incubation with MCL cell lines, clinically relevant concentrations of ibrutinib did not influence CTL019 degranulation and cytokine production (**Fig 3B**). In a 5-day proliferation assay we observed a dose-dependent reduction in T-cell proliferation and total T-cell numbers, but this reduction occurred predominantly at supra-physiological concentrations of ibrutinib (1 μM and above) and, more frequently upon the CTL019 cell exposure to JEKO-1 as compared to MCL-RL cells. (**Fig 3C and S5A**)**.** Similarly, the cell-culture supernatant analysis for 30 different cytokines demonstrated that ibrutinib did not impact cytokine production except in the presence of supra-physiological drug concentrations **(Fig 3D)**. We did not find differences in Th1/Th2 polarization between ibrutinib exposed and non-exposed CTL019 using two different techniques **(Fig 3D and S5B).** The intrinsic cytotoxic machinery of CTL019 was not significantly impacted in the presence of ibrutinib (**Fig 3E and S5C)** and there was no apparent difference in the expression of CD19 or of inhibitory ligands on MCL exposed to ibrutinib (data not shown). Notably, killing of MCL cells by CTL019 cells was significantly augmented in the presence of ibrutinib, suggesting an additive cytotoxic effect of the combination in both ibrutinib-sensitive (MCL-RL) and -resistant (JEKO-1) MCL cells (**Fig 3F**). Collectively these results indicate that ibrutinib has no adverse effect on CART cell function at physiologically relevant concentrations, and that the combination of two agents active against MCL is additive in vitro.

Impact of ibrutinib on circulating CTL019 cells

In our *in vitro* models, combination with ibrutinib clearly enhanced the already-potent anti-tumor effect of CTL019 and hence it was important to evaluate the nature of the interaction of CTL019 with ibrutinib also *in vivo*.

Inhibition of ITK has been reported to antagonize Th2 polarization and promote a Th1 phenotype. (20) However, in mice treated with CTL019 and ibrutinib we did not find an increase in Th1 cells when compared to CTL019 monotherapy (**Fig S6A**). Of note, exposure of tumor-bearing mice to ibrutinib led to an increase in peripheral blood T cells, regardless of antigen specificity, as ibrutinib augmented circulating T-cell numbers of both CTL019 and control untransduced cells (**Fig 4A** and data not shown). This increase was not due to increased proliferation, as there was no difference in the proliferation marker Ki67 between the treatment groups (**Fig 4B, left panel**). Similarly, we did not find any difference in the anti-apoptotic marker Bcl2, suggesting that the difference in the number of circulating CTL019 cells was not related to an impairment of apoptosis (**Fig 4B, right panel**). To differentiate whether the increased number of circulating T cells in ibrutinib-treated mice were due to accumulation in, or mobilization into, the peripheral blood compartment, we engrafted NSG mice with unlabeled MCL-RL cells followed by injection with luciferase-expressing T cells, wherein the bioluminescent signal (BLI) from the whole animal would correlate with total T-cell load. Ibrutinib treatment did not enhance BLI in either CTL019 or control T cell treated animals, suggesting that ibrutinib did not increase the total T-cell number but rather triggered T-cell mobilization to the blood **(Fig 4C**). We then investigated the frequency of different T-cell subsets among the circulating T cells and could not detect any difference in the T-cell subset distribution between the CTL019- and CTL019/ibrutinib- engrafted mice (**Fig**

S6B and C). Because CXCR4 is involved in ibrutinib-driven B cell mobilization in humans, we measured the expression of CXCR4 in vivo in the circulating T cells of mice treated with CTL019 or CTL019 and ibrutinib and found similar CXCR4 levels in the two groups indicating that the increased mobilization was not due to decreased CXCR4 expression **(Fig S7A).** Lastly we analyzed the expression of inhibitory/co-stimulatory receptors in the peripheral blood T cells of mice treated with CTL019 and CTL019 plus ibrutinib. There was a trend to reduced PD-1 expression when ibrutinib was added to CTL019 or untransduced T cell controls, but no differences in expression of TIM3, LAG3, CD137 or CTLA4 were found. **(Fig S7B and C).**

In vivo anti-tumor activity of ibrutinib, CTL019 and their combination

Our in vivo MCL model provided a unique opportunity to perform a direct comparison of two novel therapies that are currently used clinically as single agents. A schema of the treatment protocol is provided in **Fig 5A.** Mice treated with CTL019 showed a statistically significant improvement in lymphoma control compared to ibrutinib treated mice **(Fig 5B).** As depicted in **Fig 5C,** all mice treated with ibrutinib monotherapy died before day 100, whereas CTL019 fostered long-term survival of the recipient mice, suggesting that CTL019 is therapeutically more effective than ibrutinib in this model.

We tested next the combination of CTL019 and ibrutinib in vivo **(Fig 6A)**. Because we found no difference in anti-tumor effect when comparing untransduced T cells plus ibrutinib with ibrutinib alone **(Fig S8A)**, in all subsequent experiments the control groups were vehicle and ibrutinib alone**.** Ibrutinib monotherapy led to modestly delayed disease growth at early time points, whereas CTL019 monotherapy led to a profound reduction

in tumor burden that was followed by the disease progression beginning at 6-7 weeks. In striking contrast, 80-100% of mice treated with the combination of CTL019 and ibrutinib experienced complete, long-term disease control **(Fig 6B and C)**.

Histopathology of organs harvested at the conclusion of the experiment revealed MCL infiltrates in all untreated and ibrutinib-treated mice with the extent of involvement being relatively diminished in the ibrutinib-treated group. Most of the mice treated with CTL019 alone displayed persistent MCL and some CTL019 cells, while mice treated with CTL019-ibrutinib showed clearance of the tumor and disappearance of CTL019 **(Figure 6D).**

Having shown that ibrutinib treatment was associated with a non-significant trend to lower PD-1 expression on CTL019 in the blood compartment, we next analyzed the expression of PD-1 on CTL019 in tumor-involved organs. We confirmed the presence of T cells in the livers of mice treated with CART19 and, to a lesser extent, in CART19+ibrutinib treated mice (**Figure 6E**). Interestingly T cells from mice receiving CTL019 monotherapy had significantly higher levels of PD-1 as compared to mice receiving CTL019+ibrutinib (**Figure 6F**). We then evaluated the expression of inhibitory receptors on CTL019 cells exposed to increasing doses of ibrutinib in vitro as a possible mechanism of improved anti-tumor activity. Interestingly, we found that CTL019 cells co-cultured with MCL-RL for 6 days markedly up-regulated inhibitory receptors such as PD-1, LAG-3, TIM-3, CTLA-4 (**Figure 6G**). Notably, the addition of ibrutinib to the co-culture led to a significant reduction in all inhibitory receptors (**Figure 6G**). This mechanism may illuminate the observation of better anti-tumor activity of the combination in vitro and in vivo.

DISCUSSION

Novel therapies for B-cell malignancies include small molecule inhibitors of BCR signaling and CD19-directed T cell based therapies. The BTK inhibitor ibrutinib was recently approved by the FDA for the treatment of therapy-resistant MCL and engenders responses in most (68%) patients. However, these responses are typically partial and relatively short-lived: the median progression free survival is 17.5 months. (8) Anti-CD19 CAR T cell therapy leads to durable responses in subsets of patients with high-risk B-ALL (12-14), DLBCL (16) and, to a lesser degree, CLL. (15) Combination of chemotherapeutic agents with non-cross-resistant mechanisms of action has a long history in the treatment of cancer (33) and provides the rationale for the present study. Here we evaluated the combined effect of signal transduction (kinase) inhibition and cellular immunotherapy; these two novel therapeutic approaches are poised to revolutionize treatment of patients with lymphoma and cancer in general. Specifically, we investigated the impact of adding the BTK inhibitor ibrutinib to CTL019 using MCL as a model of a currently incurable disease responsive to both these modalities. Although ibrutinib exerted *in vitro* a profound detrimental effect on the sensitive MCL cells, we found that at all but high supra-physiological doses of the drug, CTL019 cell function remains unimpaired, with intact proliferative capacity, tumor recognition and cytotoxicity, and cytokine synthesis. This observation was not a foregone conclusion, given that at least a subset of CAR T cells expresses a tyrosine kinase that is inhibited by ibrutinib (ITK). We also demonstrated an additive effect of combining BTK signaling inhibition with the direct cytotoxicity delivered by CTL019. This finding indicates that the combined Ibrutinib and CART19 anti-MCL cell activity stems from their direct effect on the malignant B lymphocytes.

The in vitro studies were followed by a clear demonstration of superiority of CTL019 over ibrutinib in the MCL xenotransplant mouse model when each was used as monotherapy at clinically relevant doses and schedules of administration (single dose for CTL019, continuous administration for ibrutinib) and despite the fact that we used a higher dose of ibrutinib than that employed by most groups. (20) This approach is supported by our dose-titration experiments and by the fact that the dose of ibrutinib that is used in mantle cell lymphoma therapy is higher than that the one to treat CLL.

When combining ibrutinib with CTL019 in vivo, we observed complete and long-lasting tumor responses. We also noted higher numbers of circulating CTL019 cells; ibrutinib is known to lead to a peripheral blood lymphocytosis, predominantly thought to be due to mobilization of malignant B lymphocytes from lymph nodes through inhibition of CXCR4 pathway. (34-36) To our knowledge, T-cell lymphocytosis has not been formally demonstrated in patients treated with ibrutinib. Our results indicate that the T-cell lymphocytosis is not specific to antigen-specific cells, as untransduced control T cells were also shown to increase in the peripheral blood. The observed lymphocytosis does not appear to be related to increased proliferation or enhanced T-cell survival, and may be related to differential T-cell trafficking. Current data implicates CXCR4 in malignant lymphocyte trafficking in some models (35, 37) and although we did not find CXCR4 to be differentially expressed in ibrutinib-treated mice, our data do not exclude functional involvement of the CXCR4-SDF1 pathway.

Most preclinical work showing the efficacy of CTL019 has been performed using B-ALL cell lines, which are not sensitive to ibrutinib. (23) Furthermore, the strongest clinical responses to date have been obtained in patients with B-ALL, whereas patients with diffuse large B-cell lymphoma and indolent B-cell lymphomas have somewhat lower

response rates. (15) The reasons for this seemingly tumor type-specific heterogeneous responses to CTL019 remain to be elucidated.

The kinetics of the tumor response and subsequent progression suggest that ibrutinib either deepens the initial response achieved by CTL019 alone, or enhances the long-term immunosurveillance capacity of CTL019 cells. In an infectious model Dubovsky et al. (20) showed that ibrutinib enhances the percentage of antigen-specific CD8 T cells and increases the percentage of both CD4 and CD8 T cells that bear CD62L, a marker of memory T-cell differentiation. However, we did not see changes in T-cell polarization, effector function, or memory subsets in the combination therapy in our model; if found, these would have pointed towards immunological memory as a potential mechanism of action. The most stringent test for initiation of memory is by tumor re-challenge in animals that have cleared disease. However, in this model the only animals that successfully clear tumor long-term are those who received the combination therapy and therefore there is not a suitable control group with which to compare. Therefore, the exact mechanism(s) of the strong anti-lymphoma effect of the CTL019/ibrutinib combination remains to be elucidated but most likely reflects the advantage of simultaneous direct targeting of malignant cells with two therapeutic modalities with vastly different modes of action. The observation that T cells, including CTL019 cells, are mobilized into the peripheral blood may also help to explain the augmented anti-tumor effect that we observed.

Recently, ibrutinib has been found to enhance the anti-tumor effect of blockade of the PD1/PD-L1 system in mouse models (38), a phenomenon that was accompanied by enhanced anti-tumor immune responses. These authors did not show reduction of PD1 or PD-L1 molecules upon exposure to ibrutinib. In contrast, here we found that tumor-

389 infiltrating CTL019 cells had lower PD-1 expression if the animals were also treated with
390 ibrutinib and these results were further corroborated by in vitro studies showing that
391 exposure to MCL cells led to a marked increase in inhibitory receptors ("immune
392 checkpoint molecules") on CTL019 that was partially abrogated by co-treatment with
393 ibrutinib. These observations may suggest that this two-pronged anti-tumor approach
394 derives additional synergy from ibrutinib-mediated T cell mobilization and from ibrutinib-
395 mediated reduction in inhibitory receptor expression on CART cells.

396 Regardless of the above uncertainties, this is the first pre-clinical study that combines
397 signal transduction inhibition with adoptive T-cell immunotherapy by targeting BTK and
398 CD19, respectively. Our findings document a potent additive therapeutic effect of this
399 novel and highly promising combination acting by enhanced killing of the MCL cells.
400 They also pave the way for clinical trials of this and similar non-cross resistant
401 combinations in patients with MCL and other types of B-cell lymphoma.

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AUTHORSHIP CONTRIBUTIONS

M.R., S.S.K., S.G., M.Ka., M.A.W., C.H.J. formulated the initial ideas and planned the experiments. M.R., S.S.K., O.S., M.V.M., J.A.F., Q.Z., X.L., O.U.K, M.M, S.N.C., M.KI. performed the experiments, analyzed the data, and contributed to the manuscript. S.Q. and M.A.W. reviewed the pathology slides. A.M. and S.J.S. provided the primary samples from MCL patients. S.G., M.A.W. and M.R. wrote the manuscript and C.H.J edited. S.F.L. performed the Luminex assays. All the authors reviewed and accepted the contents of the article.

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527

FIGURE LEGENDS

Figure 1. Establishment of an ibrutinib-sensitive MCL cell line. A. Morphology, phenotype and FISH analysis of the MCL-RL cell line and primary cells. Thin-layer cell preparation of the MCL-RL cell line was obtained by Cytospin. MCL-RL cells were stained with Giemsa and demonstrated a blastoid morphology (top, left panel). Flow cytometry analysis revealed that CD19 and CD5 co-expression, hallmark of MCL, is maintained (right panels). At FISH analysis, MCL-RL cells were analyzed by FISH using a dual color gene fusion probe against the IgH (green) and CCND1 (orange) genes, located on chromosomes 14 and 11, respectively. The isolated green color corresponds to the non-translocated IgH gene locus and isolated orange to the CCND1 gene locus. The fused green and orange, typically blended together into a yellow color, mark the translocated, hybrid IgH/CCND1 gene (bottom left panel). **B. MTT assay of MCL cell lines**. JEKO-1, MINO and MCL-RL were cultured for 48 hours with increasing doses of ibrutinib (0-10 μM). MCL-RL cell line was the most sensitive to ibrutinib, with an IC50 of 10 nM. The MCL cell lines MINO and JEKO-1 are more resistant. **C. Ibrutinib-sensitivity of MCL-RL cell line in vivo.** NSG mice were engrafted with luciferase-positive MCL-RL cells ($1x10^6$/mouse); at day 7 mice were randomized according to tumor burden (bioluminescence, BLI) to receive vehicle (HP-beta-cyclodextrin), ibrutinib 25 mg/Kg/day or ibrutinib 125 mg/Kg/day in the drinking water. A dose-related anti-lymphoma activity was observed using bioluminescence (BLI, top panel) (ANOVA at day 70 $p<0.0001$ for both doses). This anti-lymphoma activity was also reflected in an improved overall survival of mice treated with both doses compared to controls (Log-Rank test $p=0.0086$ and 0.0017 respectively) (bottom panel). Graphs are representative of 2 experiments with 4-5 animals per group.

552 **Figure 2. CTL019 cells exhibit potent in vitro and in vivo effector functions**
553 **against diverse MCL cell lines. A. Feasibility of CTL019 production in MCL**
554 **patients and anti-lymphoma effector activity.** PB (#001) or BM (#002) samples were
555 obtained from patients with active MCL (infiltration 68% and 4% respectively). CAR19 T
556 cells were expanded according to the standard protocol used at our institution (see
557 methods). CTL019 expansion was feasible for both PB and BM T cells, with a range of
558 population doublings from 3.5 (BM#002) to 6.5 (PB#001). Expanded T cells included
559 both CD8 and CD4 cells with a variable CAR19 expression (from 10% to 49%) similar to
560 what is currently obtained in other CTL019 trials at our institution (top panel). (28) As
561 shown in the bottom panel, CTL019 and MCL cells from the same patient were co-
562 cultured for 6 hours and then harvested and analyzed by flow cytometry for CD107a
563 degranulation or cytokine production. Autologous CTL019 but not control T cells (UTD)
564 showed significant activation with CD107a degranulation and intra-cytoplasmic
565 production of cytokines, including IL-2, TNFa, IFNg, GM-CSF and MIP1b. **B. CD107a**
566 **degranulation assay.** CAR19+ T cells showed specific CD107a degranulation when
567 co-cultured with JEKO-1 and MCL-RL MCL cell lines, similar to the positive control PMA
568 and ionomycin stimulation (PI). **C. CTL019 cytotoxicity and proliferation assays.** For
569 the cytotoxicity assay (left panel) CTL019 were co-cultured at different effector-to-target
570 ratio (E:T) with luciferase-positive MCL cell lines or control (K562). At 24 hours cell
571 killing was assessed by luminescence relative to controls. CTL019 are able to induce
572 cell death in both MCL cell lines (1way ANOVA significant at all ratios > 0:1 compared to
573 control cell line K562) with a dose correlation effect; no cytotoxicity is observed against
574 the CD19-negative control cell line (K-562). For the proliferation assay (right panel)
575 CFSE-labeled CTL019 cells were co-cultured with the MCL cell lines (JEKO-1, MCL-RL)

or control (K562) for 5 days. CTL019 show specific proliferation (CFSE dilution) when co-cultured with MCL cell lines but not with control. **D. In vivo potent anti-lymphoma activity of CTL019 against both ibrutinib-resistant (JEKO-1) and ibrutinib sensitive cell line (MCL-RL).** NSG mice were engrafted with either luciferase-positive JEKO-1 cells (top panel) or MCL-RL (bottom panel). At day 6 mice were randomized to receive 3 different doses of CTL019 ($0.5x10^6$, $1x10^6$, $2x10^6$/mouse, CAR+ 70%) or control T cells (UTD, $1x10^6$/mouse). A dramatic anti-lymphoma activity was observed in all doses, with the highest dose ($2x10^6$), leading to long term complete remission in JEKO-1 (ANOVA at day 28 $p<0.0001$ for all CTL019 doses). In MCL-RL luminescence values are shown at 1 week after T cell infusion; a significant dose-dependent CTL019 anti-lymphoma activity is observed (1way ANOVA $p<0.05$ for the doses of $1x10^6$ and $2x10^6$ CATL019/mouse). In the MCL-RL model late relapses are observed, also at the dose of $2x10^6$ CART19/mouse (data not shown). Each graph is representative of 2 independent experiments, each with 4-5 mice per group.

Figure 3. Impact of ibrutinib on CTL019 functions in vitro. A. Western blot analysis of p-ITK inhibition by ibrutinib in CAR19 cells. CTL019 cells were stimulated with anti-CD3/CD28 Dynabeads or anti-CAR19 beads for 2 minutes in the presence of different concentrations of ibrutinib (10-1000 nM) or control (DMSO). Western blot analysis on protein lysates revealed modest inhibition of the phosphorylation of ITK (at Y_{180}) particularly in CAR stimulated CTL019 at the highest concentration (1 μM). Beta-actin and p-Erk were used as loading and activation controls respectively. **B. CTL019 Degranulation assay and cytokine production in the presence of ibrutinib.** CTL019 or control T cells (UTD) were co-cultured with JEKO-1 or MCL-RL for 4 hours in the presence of increasing doses of ibrutinib (10-1000 nM).

Positive (PMA/ionomycin) and negative controls (media alone, K562) were also included. Flow cytometric analysis revealed significant activation of CTL019 cells but not UTD in the presence of MCL cell lines as shown by CD107a degranulation and intra cytoplasmic cytokine production (IL-2, TNFa). **C. CTL019 proliferation assay in the presence of ibrutinib.** CFSE labeled CTL019 cells were co-cultured with lethally irradiated MCL-RL cells or JEKO-1 (or controls, K562) for 5 days in the presence of increasing doses of ibrutinib (10-1000 nM, added at every change of media). Cells were then analyzed for CFSE dilution, as a marker of cell proliferation.. Profound CTL019 proliferation was observed, however significant reduction in CFSE positive T cells was observed with the highest (supra-physiological) ibrutinib doses (1000 nM ibrutinib) in the JEKO-1 group. **D. CTL019 cytokine production in the presence of ibrutinib.** CTL019 or control T cells were co-cultured with irradiated MCL-RL cells for 3 days and supernatants were analyzed for 30 human cytokines (Luminex, 30-plex). Intense production of both Th1 and Th2 cytokines was observed with significant reduction of all cytokines at the highest ibrutinib dose (1000 nM). **E. Effect of ibrutinib on CTL019 cytotoxic machinery.** CTL019 were expanded with anti-CD3/CD28 beads in the presence of increasing doses of ibrutinib (10-1000 nM). RT-PCR analysis of Fas ligand, granzyme B, perforin and TRAIL mRNA expression was performed at the end of expansion (day 10). No clear effect of increasing doses of ibrutinib was observed (1way ANOVA= ns). A trend in increased perforin expression was not statistically significant. **F. CTL019 cytotoxicity in the presence of ibrutinib.** CTL019 or control T cells (UTD) were co-cultured at different effector-to-target ratio (E:T) with luciferase-positive MCL cell lines (JEKO-1, MCL-RL) with increasing doses of ibrutinib. At 24 hours cell killing was assessed by luminescence. CTL019 are able to induce cell death in both MCL cell

lines. At a specific E:T ratio, increased MCL killing was significantly correlated to increased ibrutinib dose. The p-values (1way ANOVA) comparing CART19-DMSO vs. CART19 +IBRU 100 nM at the different E:T ratios are summarized in the figure.

Figure 4. Increase in circulating CTL019 cells in the presence of Ibrutinib. A. Higher number of circulating CAR19 T cells in the combination treatment. Peripheral blood (PB) circulating T cells were monitored weekly by retro-orbital bleeding and flow cytometry analysis was performed. Expansion of CTL019 in the periphery was detected in both CTL019 and CTL019-ibrutinib (ibrutinib 125mg/Kg/day in the drinking water) treated mice; however, a significantly higher number of T cells was observed in the combination group (Student's t-test). Peak expansion is usually observed 1-2 week after T cell infusion. **B. In vivo T cell proliferation and apoptosis after treatment with CTL019/ibrutinib combination.** One week after CTL019 infusion in MCL-RL bearing mice, PB was collected and analyzed for Ki67 and bcl-2 by flow cytometry. No statistically (Student's t-test) significant difference in T cell proliferation (Ki67) or apoptosis (bcl-2) was observed. **C. In vivo tracking of T cell expansion.** NSG mice were engrafted with WT MCL-RL cells. After one month luciferase positive CTL019 or control T cells were infused. Five days after infusion mice were analyzed by bio-luminescence imaging. A significant increase in T cell number was observed in both CTL019 and CTL019-ibrutinib treated mice as compared to control T cells (UTD) and UTD-ibrutinib. No difference in T cell proliferation was detected between CTL019 and CTL019-ibrutinib (Student's t-test).

Figure 5. Direct comparison of the anti-MCL activity of ibrutinib and CTL019 in MCL xenografts. A. Protocol schema. NSG mice were engrafted with luciferase-positive MCL-RL cells ($2x10^6$ cells/mouse, i.v.). At day 7 mice were randomized according to tumor burden, to receive vehicle, ibrutinib 125 mg/Kg/day or CTL019 $2x10^6$/mouse. Ibrutinib and vehicle were continued for all the duration of the experiment. **B. and C. CTL019 therapy is more effective than ibrutinib against MCL-RL.** Mice treated with CTL019 had a significantly improved anti-tumor activity compared to ibrutinib (Student's t-test, $p<0.0001$ from day 18). CTL019 treatment ensured also a statistically improved overall survival compared to ibrutinib (Log-Rank test, $p<0.005$) **(C)**. Graphs are representative of 2 experiments, each with 5 mice per group; p-values compared to ibrutinib alone. The dotted bar represents the limit of detection.

Figure 6. Combination of ibrutinib and CTL019 in MCL xenografts. A. Protocol schema. NSG mice were engrafted with luciferase-positive MCL-RL cells ($2x10^6$ cells/mouse, i.v.). At day 7 mice were randomized according to tumor burden, to receive vehicle, ibrutinib 125 mg/Kg/day, CTL019 $2x10^6$/mouse or CTL019 with ibrutinib (same doses). Ibrutinib and vehicle were continued for all the duration of the experiment. **B and C. Increased anti-lymphoma activity of the CTL019-ibrutinib combination.** Mice treated with CTL019 in combination with ibrutinib displayed a significantly better anti-lymphoma effect compared either ibrutinib (Student's t-test, $p<0.0001$ at day 60) or CTL019 alone ($p=0.007$ at day 110). At long term follow up 5/5 mice in the ibrutinib group and 4/5 mice in the CTL019 group are progressing while only 1 mouse in the CTL019-ibrutinib combination has progressed. Graphs are representative of 2 experiments, each one with 5 mice per group. The dotted bar represents the limit of detection. The bioluminescence images of 2 representative mice per group are shown

in panel **C.** Please note that the range of radiance for visualization varies among different time points, as detailed in the figure. **D. Mouse liver histopathology after treatment with CTL019/ibrutinib combination.** Animals were euthanized at the end of the experiment (day 120) or when required according to animal welfare regulations; organs (liver, spleen, bone marrow) were collected for histopathology (H&E, PAX5, CD2). Representative mice are shown in the figure. Variable amount of disease is observed in the liver of untreated mice, and ibrutinib mice and CTL019 treated mice; most of mice treated with CTL019-ibrutinib combination had no disease. CD2+ T cells were detected in CTL019 mice (at the time of progression, together with MCL-RL cells) while CTL019-ibrutinib treated mice showed disappearance of T cells. The livers of these mice were analyzed by flow cytometry (panel **E**) and residual T cells showed differential expression of PD-1: PD-1 was significantly upregulated in mice not receiving ibrutinib (panel **F**), possibly explaining the lack of anti-tumor activity. **G. Co-culture of CTL019 with MCL-RL cell line leads to overexpression of inhibitory receptors and this overexpression is reduced in the presence of ibrutinib.** CART19 cells were co-cultured with MCL-RL cells in the presence or not of ibrutinib (100 nM). At day 6 inhibitory receptor expression (PD-1, LAG-3, TIM-3 and CTLA-4) was analyzed by flow cytometry. Marked upregulation of inhibitory receptor In T cells is observed. However, a significant reduction in the surface expression of PD-1, LAG-3, TIM-3 and CTLA-4 was detected when CART19 cells were cultured with ibrutinib 100 nM.

Figure 1

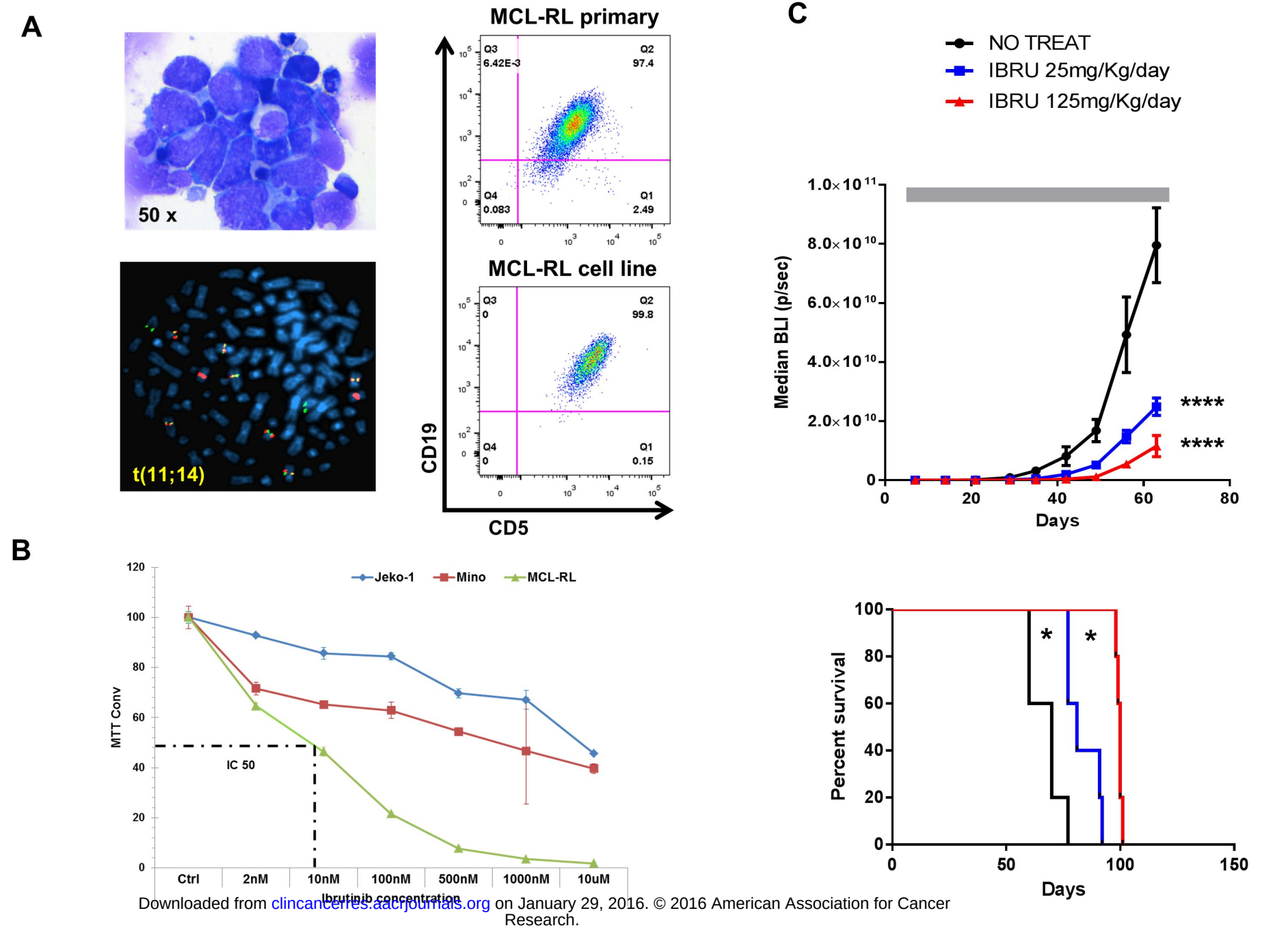

Figure 2 Author Manuscript Published OnlineFirst on January 27, 2016; DOI: 10.1158/1078-0432.CCR-15-1527
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

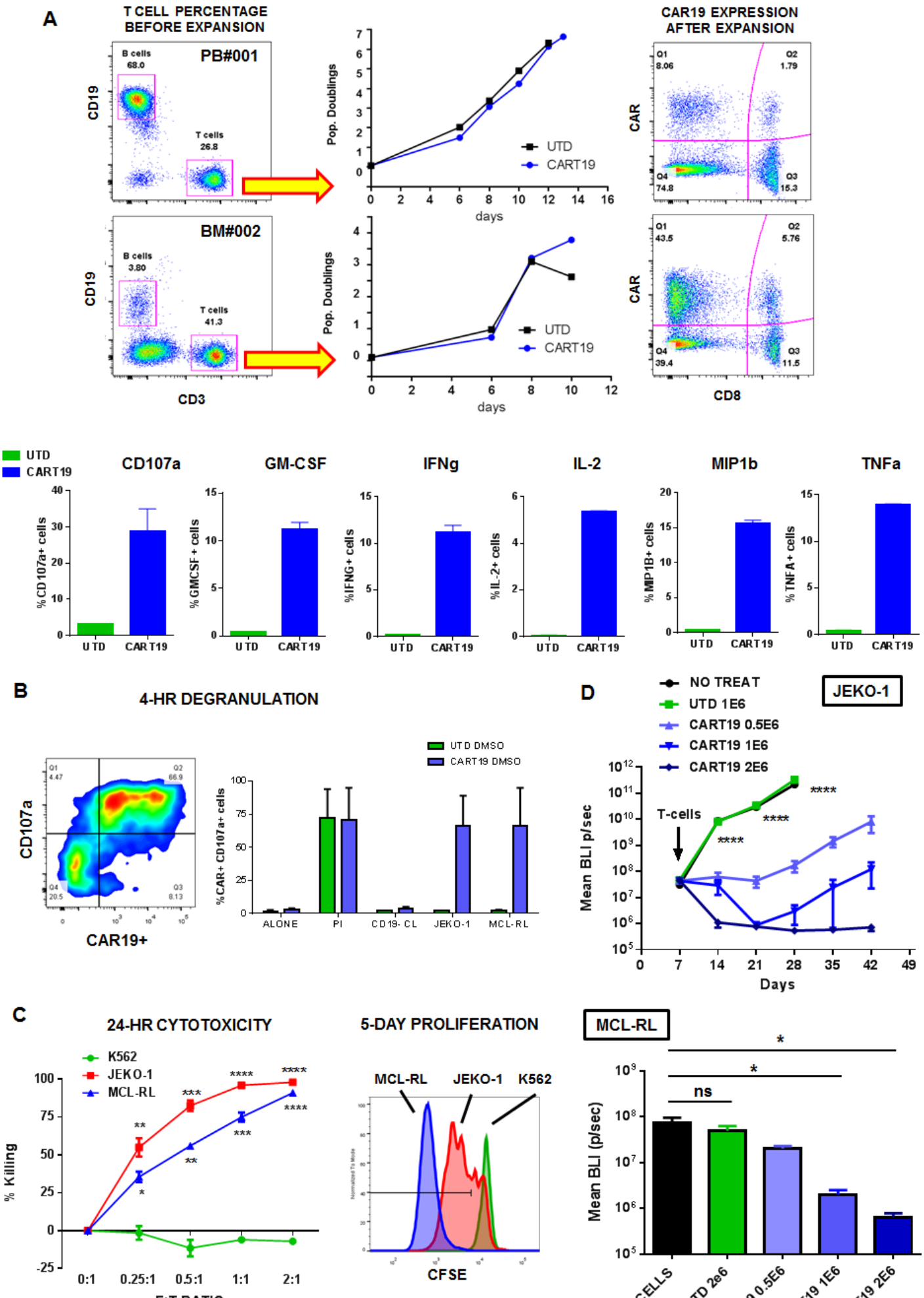

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Figure 3

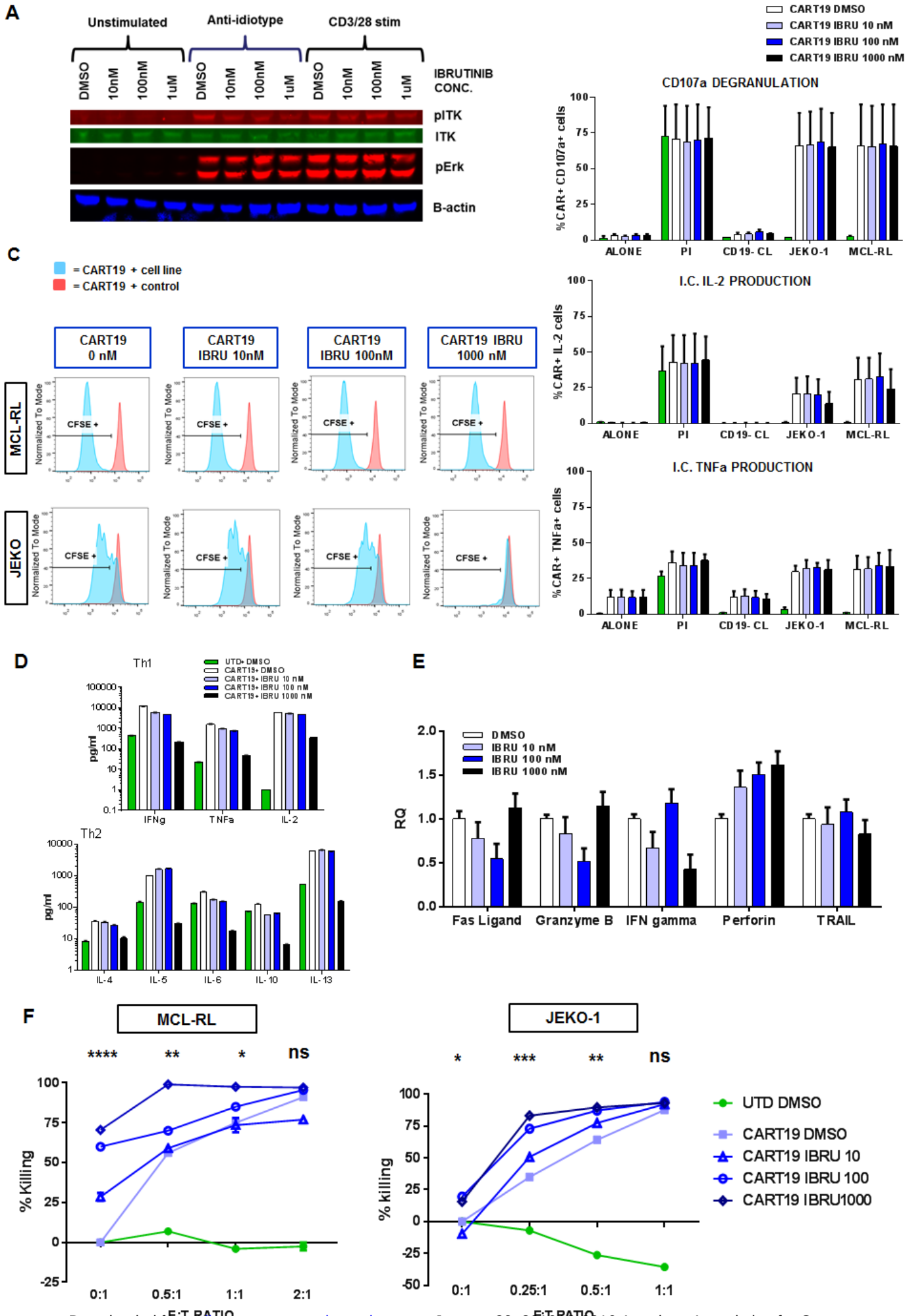


Figure 4

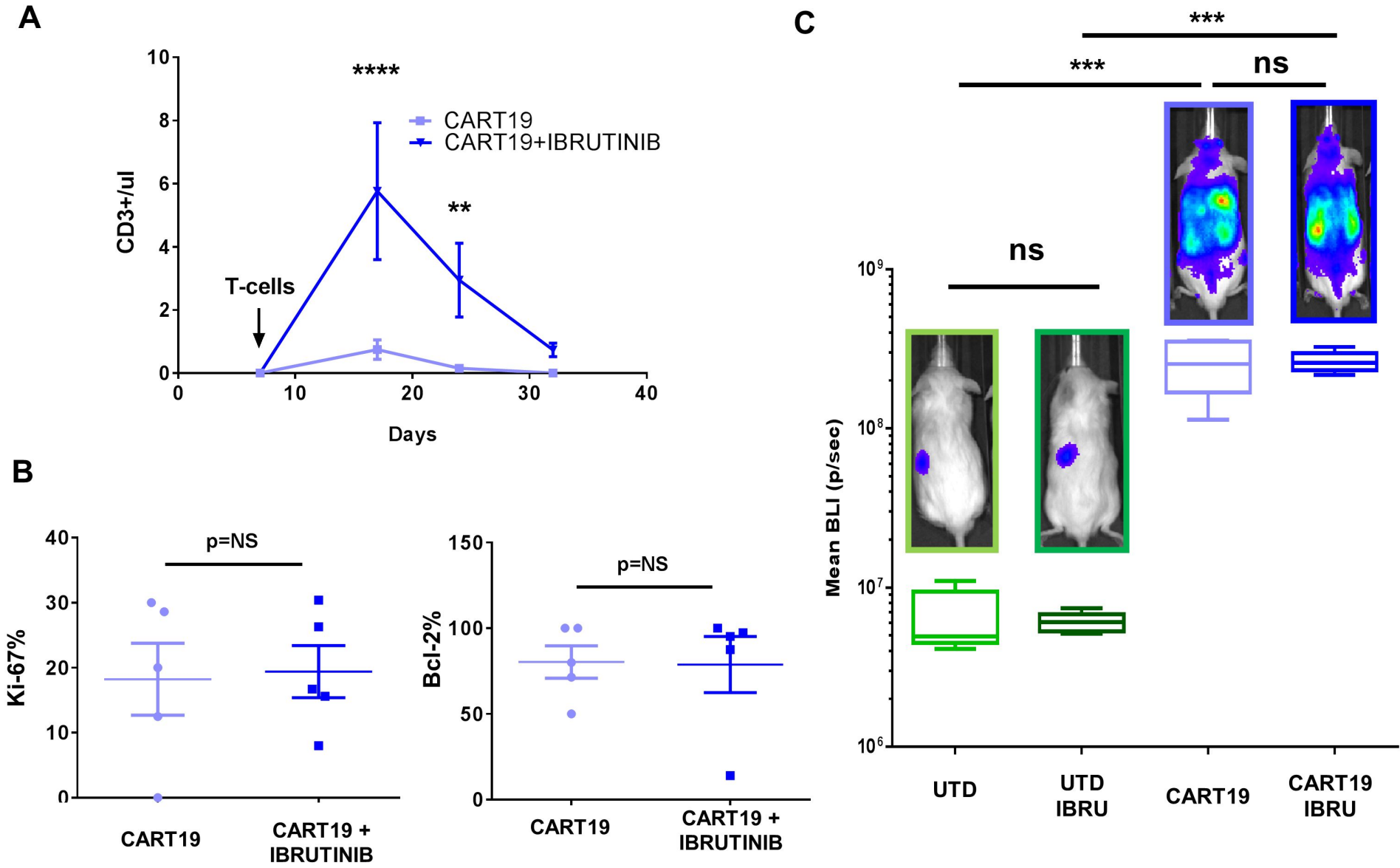


Figure 5

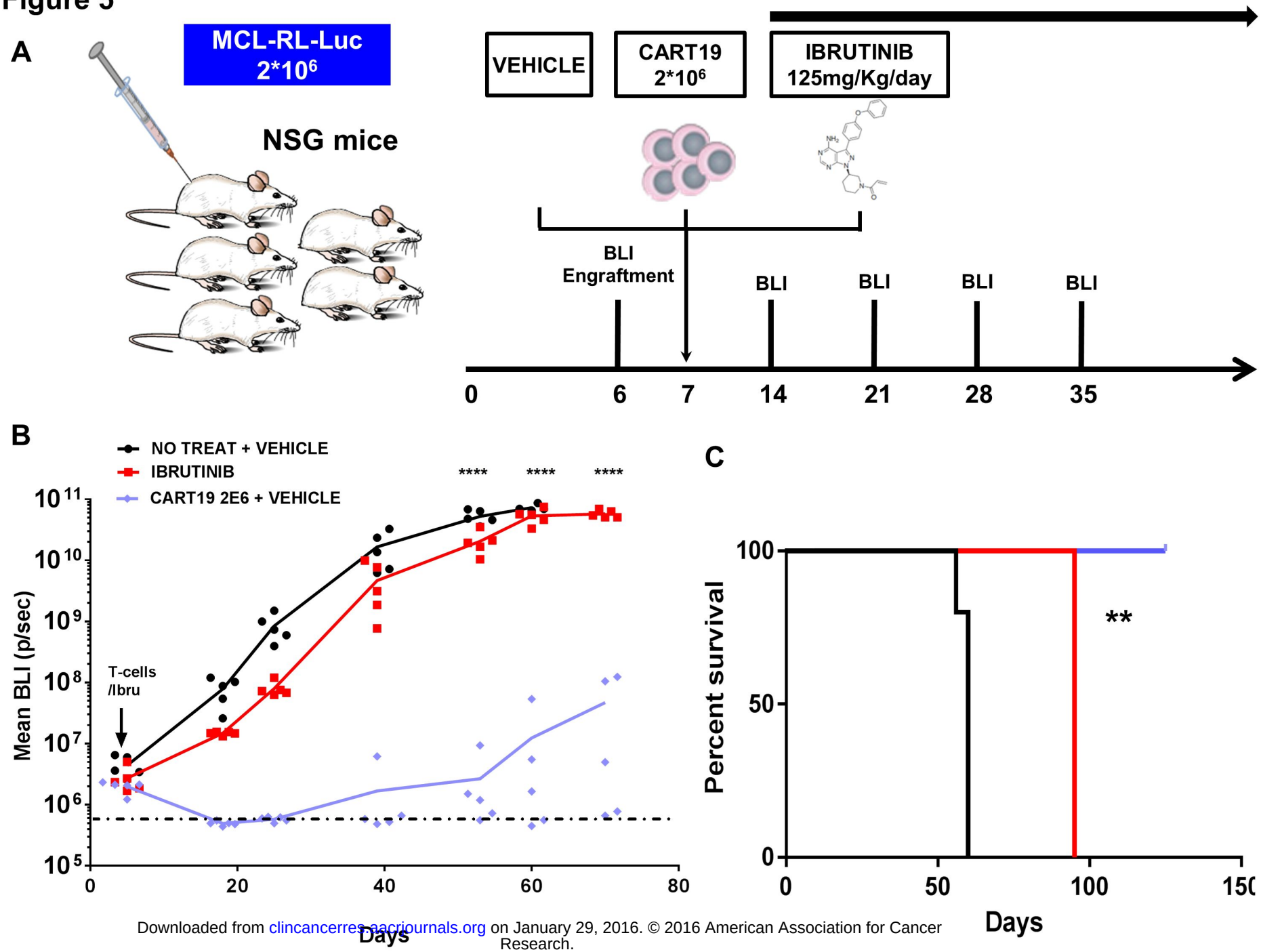
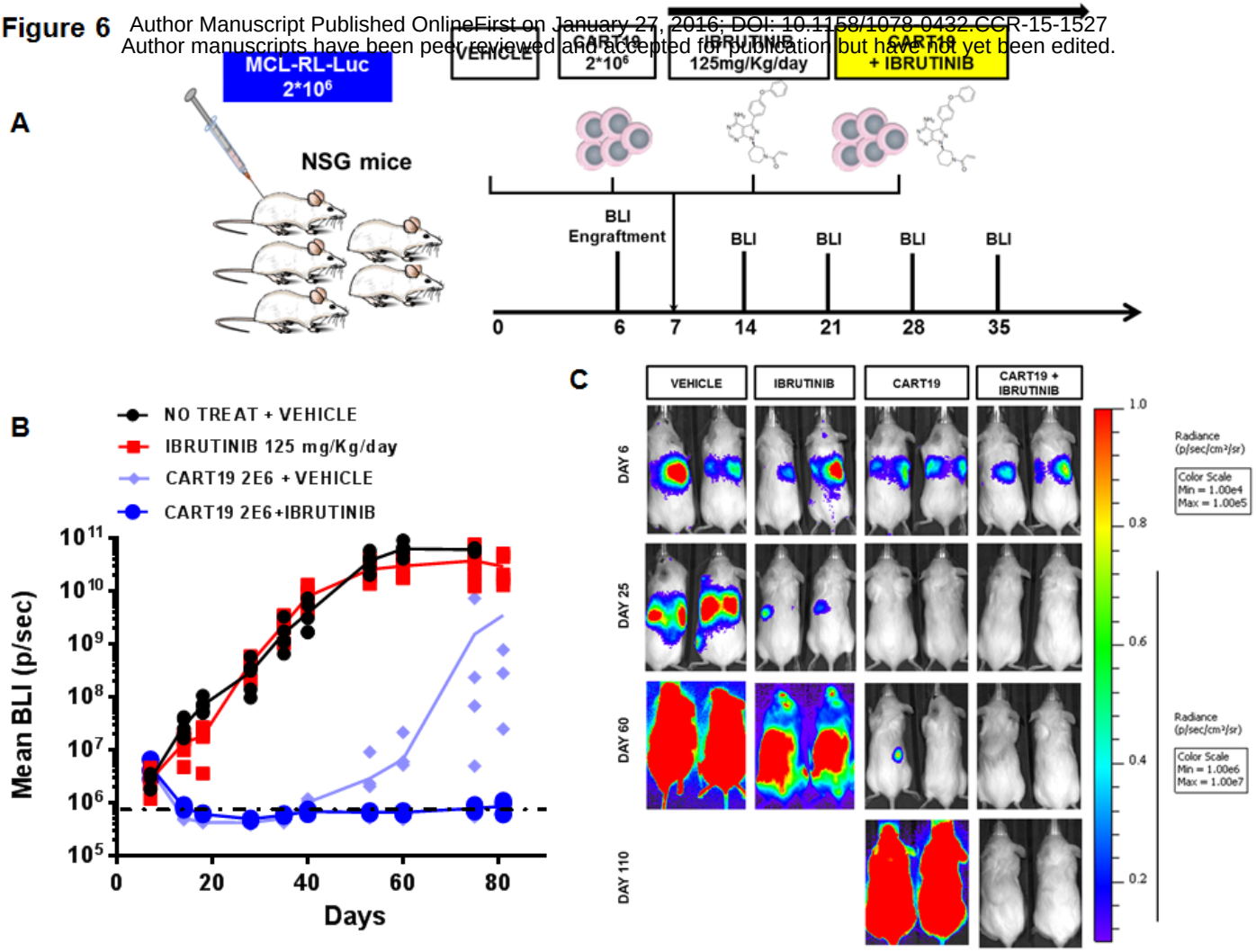


Figure 6



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The Addition of the BTK inhibitor Ibrutinib to Anti-CD19 Chimeric Antigen Receptor T Cells (CART19) Improves Responses against Mantle Cell Lymphoma

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