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 with ibrutinib-treated mice (median survival not reached vs. 95 days, P < 0.005) but most mice receiving CTL019 monotherapy eventually relapsed. Therefore, we added ibrutinib to CTL019 and showed that 80% to 100% of mice in the CTL019 + ibrutinib arm and 0% to 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.

Cancer Therapy: Preclinical

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 4 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, as 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 antilymphoma 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 antitumor 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 the activation of T lymphocytes (19). Recent preclinical data suggest that ibrutinib preferentially inhibits Th2-polarized CD4 T cells thus skewing T cells towards Th1 antitumor immune response (20). However, another recent

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.

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 blastoid morphology (top left). Flow cytometry analysis revealed that CD19 and CD5 coexpression, hallmark of MCL, is maintained (right). In 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 nontranslocated 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). 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 μmol/L). MCL-RL cell line was the most sensitive to ibrutinib, with an IC50 of 10 nmol/L. The MCL cell lines MINO and JEKO-1 were more resistant. C, ibrutinib sensitivity of MCL-RL cell line in vivo. NSG mice were engrafted with luciferase-positive MCL-RL cells (1 × 106/mouse); at day 7 mice were randomized according to tumor burden (bioluminescence, BLI) to receive vehicle (HP-b-cyclodextrin), ibrutinib 25 mg/kg/day, or ibrutinib 125 mg/kg/day in the drinking water. A dose-related antilymphoma activity was observed using bioluminescence (top; ANOVA at day 70, P < 0.0001 for both doses). This antilymphoma activity was also reflected in an improved overall survival of mice treated with both doses compared with controls (log-rank test P = 0.0086 and 0.0017, respectively; bottom). Graphs are representative of two experiments with 4–5 animals per group.
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 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 antitumor effect and leads to prolonged remissions.

Materials and Methods
Cell lines and primary samples
Cell lines were originally obtained from ATCC (K-562, Mino and K562-130-1) or DSMZ (MOLM-14 and NALM-6). T cell lines were obtained more than 6 months prior experiments and authentication was performed by cell banks utilizing short tandem repeat profiling. 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). For some experiments, MCL-RL and JEKO-1 cells were transduced with firefly luciferase/eGFP and then sorted to obtain >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 RPMI1640 (Gibco, 11875–085, LifeTechnologies) supplemented with 10% FBS (Gemini, 100-085, LifeTechnologies) and 50 U/mL penicillin/streptomycin (Gibco, Life Technologies, 15070–063). Deidentified primary human MCL bone marrow and peripheral blood specimens were obtained from the clinical practices of University of Pennsylvania under an Institutional Review Board–approved protocol (UPCC #03409). For all functional studies, primary cells were thawed at least 12 hours before experiment and rested at 37°C.

FISH and IHC
The FISH analysis and IHC were performed according to the standard method and as described previously (22). Specifics of the experiment of this article are detailed in the Supplementary Methods section.

IHC
Thin-layer cell preparation was obtained by Cytospin (Thermo Scientific) and stained with Giemsa. For formalin-fixed paraffin-embedded tissues, immunohistochemical staining was performed on a Leica Bond-III instrument (Leica Biosystems) 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 human anti-CD19 CAR transmembrane domain (CD8 hinge, 4-1BB costimulatory domain and CD3 zeta signaling domain) was generated as described previously (ref. 23; Supplementary Fig. S3A). Production of CAR-expressing T cells was performed as described previously (ref. 24; Supplementary Fig. S3B).

Ibrutinib
Ibrutinib (PCI-32765) was purchased from Medkoo (#202171) or Selleck Biochemicals (#S62680) 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 1,000 nmol/L in culture media. For in vivo experiment, ibrutinib powder was dissolved in a 10% HP-β-cyclodextrin solution (1.6 mg/mL) and administered to mice in the drinking water.
**Multimetric flow cytometry**

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

**MTT enzymatic conversion assay**

The assay was performed as described previously (26). Specifications of this experiment are detailed in the Supplementary Methods section.

**DNA fragmentation (TUNEL) assay**

ApoAlert DNA fragmentation assay kit (Clontech, 630108) was used according to the manufacturer’s protocol. In brief, cells were cultured at 0.5 × 10^6 cells/mL for 72 hours with DMSO (control) or ibrutinib at the listed doses. The cells were then washed, fixed, permeabilized, and incubated for 1 hour at 37°C with or without terminal deoxynucleotidyl transferase (TdT). After exposure to the stopping buffer and washing, the cells were analyzed by flow cytometry using the CellQuest PRO software v. 5 (BD Biosciences).

**Western blot analysis**

The assay was performed as described previously (27). Specifications of this experiment are detailed in the Supplementary Methods section.

**Real-time PCR**

CTL019 cells were screened by RT-PCR analysis for Fas ligand (Applied Biosystems, Life Technologies, Hs00181225_m1), granzyme B (Applied Biosystems, Hs001554355), perforin (Applied Biosystems, Hs00169473_m1), and TRAIL (Applied Biosystems, Hs00921974_mRNA expression at the end of expansion (day 10)). RNA was extracted with RNAqueos-4PCR Kit (Ambion, Life Technologies, AM-1914) and cDNA was synthesized with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, 170-8841). The relative target cDNA copies were quantified by quantitative PCR (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, carboxyfluorescein diacetaoeuccinimidyI ester proliferation (CFSE), cytotoxicity assays, and cytokine measurements were performed as described previously (24,28).

Specifications of this experiment are detailed in the Supplementary Methods section.

**Animal experiments**

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

**Statistical analysis**

All statistical analyses were performed as indicated using GraphPad Prism 6 for Windows, version 6.04. Student t test was used to compare two groups; in analysis where multiple groups were compared, one-way ANOVA was performed with Holm–Sidak correction for multiple comparisons. When multiple groups at multiple time points/number of comparisons this experiment are displayed 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 polyomorphic morphology, the characteristic MCL immunophenotype including CD19 and CD5 coexpression, and the classical t(11;14) translocation with 6 to 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 10 nmol/L (Fig. 1B), including their apoptotic cell death (Supplementary 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 and 10 μmol/L, respectively (Fig. 1B) and showed no evidence of cell death (Supplementary 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 (Supplementary 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 μmol/L, respectively (Supplementary Fig. S1C) and hence served as additional negative controls throughout the study. To determine the suitability of MCL-RL cells for in vivo experiments, we injected immunodeficient NSG mice intravenously 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 bioluminescence imaging. A significant increase in T cell number was observed in both CTL019 and CTL019-ibrutinib treated mice as compared with control T cells (UTD) and UTD-ibrutinib. No difference in T-cell proliferation was detected between CTL019 and CTL019-ibrutinib (Student t test). BLI, bioluminescence.

Figure 4.
Increase in circulating CTL019 cells in the presence of ibrutinib (IBRU). 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 125 mg/kg/day in the drinking water) treated mice; however, a significantly higher number of T cells was observed in the combination group (Student t test). Peak expansion is usually observed at 1–2 weeks 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, peripheral blood was collected and analyzed for Ki67 and bcl-2 by flow cytometry. No statistically (Student 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 bioluminescence imaging. A significant increase in T cell number was observed in both CTL019 and CTL019-ibrutinib treated mice as compared with control T cells (UTD) and UTD-ibrutinib. No difference in T-cell proliferation was detected between CTL019 and CTL019-ibrutinib (Student t test). BLI, bioluminescence.

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 the clinical trials of our group (16) and used for the following experiments. The design of this CAR and the T-cell production schema are shown in Supplementary Fig. S3A and S3B. 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) and then performed a CD107a degranulation and cytokine production assay to demonstrate reactivity against that patient’s own MCL (Fig. 2A, bottom).

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 Supplementary Fig. S4A and S4B). 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 and C and Supplementary Fig. S4C). As shown in Fig. 2C, the MCL-RL cell line was less sensitive to CTL019 cytotoxicity as compared with JEKO-1. This was likely due to the increased activation-induced apoptosis of CTL in the presence of MCL-RL (Supplementary Fig. S4D). The CTL019 activation was strictly CAR-dependent, as 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 antitumor efficacy, with $2 \times 10^6$ CTL019 cells per mouse proving to be the most effective. Higher doses of T cells were associated with nonspecific alloreactivity (data not shown). Notably, the antilymphoma activity of CTL019 was observed in NSG mice engrafted with both ibrutinib-resistant (JEKO-1; Fig. 2D, top) and ibrutinib-sensitive (MCL-RL) MCL cell lines (Fig. 2D, bottom). 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 CD T cells as a suppressor of the 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, IFN-γ, TNF) and Th2-type (IL4, IL5, IL10), 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 100–150 ng/mL; ref. 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 the 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 to 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 supraphysiologic concentrations of ibrutinib (1 μM/L and above) and, more frequently, upon the CTL019 cell exposure to JEKO-1 as compared with MCL-RL cells. (Fig. 3C and Supplementary Fig. S5A). Similarly, the cell–culture supernatant analysis for 30 different cytokines demonstrated that ibrutinib did not impact cytokine production except in the presence of supraphysiologic drug concentrations (Fig. 3D). We did not find differences in Th1/Th2 polarization between ibrutinib exposed and nonexposed CTL019 using two different techniques (Fig. 3D and Supplementary Fig. S5B). The intrinsic cytotoxic machinery of CTL019 was not significantly impacted in the presence of ibrutinib (Fig. 3E and Supplementary Fig. 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 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 antitumor 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 with CTL019 monotherapy (Supplementary 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). Similarly, we did not find any difference in the ant 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). 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. Supplementary Fig. S6D and S6E). Because CXC4 is involved in ibrutinib-driven B-cell mobilization in humans, we measured the expression of CXC4 in vivo in the circulating T cells of mice treated with CTL019 or CTL019 and ibrutinib and found similar CXC4 levels in the two groups indicating that the increased mobilization was not due to decreased CXC4 expression (Supplementary Fig. S7A). Finally, we analyzed the expression of inhibitory/costimulatory 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. (Supplementary Fig. S7B and S7C).

In vivo antitumor 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 with 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.

Figure 6. Combination of ibrutinib and CTL019 in MCL xenografts. A, protocol schema. NSG mice were engrafted with luciferase-positive MCL-RL cells (2 × 10⁶ cells/mouse, i.v.) at day 0. At day 7, mice were randomized according to tumor burden, to receive vehicle, ibrutinib 125 mg/kg/day, CTL019 2 × 10⁶/mouse, or CTL019 with ibrutinib (IBRU; same doses). Ibrutinib and vehicle were continued for all the duration of the experiment. B and C, increased antilymphoma activity of the CTL019–ibrutinib combination. Mice treated with CTL019 in combination with ibrutinib displayed a significantly better antilymphoma effect compared either with 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 of 5 mice in the ibrutinib group and 4 of 5 mice in the CTL019 group are progressing while only 1 mouse in the CTL019–ibrutinib combination group showed no disease. 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. CD2³ 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 (E) and residual T cells showed differential expression of PD-1. PD-1 was significantly upregulated in mice not receiving ibrutinib (F), possibly explaining the lack of antitumor activity. G, coculture 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 cocultured with MCL-RL cells in the presence or not of ibrutinib (100 nmol/L). 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 nmol/L.
of the recipient mice, suggesting that CTL019 is therapeutically more effective than ibrutinib in this model.

We next tested the combination of CTL019 and ibrutinib in vivo (Fig. 6A). Because we found no difference in the antitumor effect when comparing untransduced T cells plus ibrutinib with ibrutinib alone (Supplementary 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% to 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 (Fig. 6D).

Having shown that ibrutinib treatment was associated with a nonsignificant trend to lower PD-1 expression on CTL019 in the blood compartment, we next analyzed the expression of PD-1 on 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 (Fig. 6E). Interestingly, T cells from mice receiving CTL019 monotherapy had significantly higher levels of PD-1 as compared with mice receiving CTL019 + ibrutinib (Fig. 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 antitumor activity. Interestingly, we found that CTL019 cells cocultured with MCL-RL for 6 days marked upregulated inhibitory receptors such as PD-1, LAG-3, TIM-3, CTLA-4 (Fig. 6G). Notably, the addition of ibrutinib to the coculture led to a significant reduction in all inhibitory receptors (Fig. 6G). This mechanism may illuminate the observation of better antitumor 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 chemotherapy agents with non–cross-resistant mechanisms of action has a long history in the treatment of cancer (33) and provides the rationale for the current 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 all but high supraphysiologic 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 MCL therapy is higher than that of 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 implicate 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 and colleagues (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 toward immunologic
memory as a potential mechanism of action. The most stringent test for initiation of memory is by tumor rechallenge in animals that have cleared disease. However, in this model, the only animals that successfully cleared 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 antilymphoma 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 antitumor effect that we observed.

Recently, ibrutinib has been found to enhance the antitumor effect of blockade of the PD1/PD-L1 system in mouse models (38), a phenomenon that was accompanied by enhanced antitumor 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-infiltrating CTL019 cells had lower PD-1 expression if the animals were also treated with ibrutinib. These observations may suggest that this two-molecules (“免疫 checkpoint molecules”) on CTL019 that was partially abrogated by cotreatment with ibrutinib. These observations may suggest that this two-pronged antitumor approach derives additional synergy from ibrutinib-mediated T-cell mobilization and from ibrutinib-mediated reduction in inhibitory receptor expression on CAR T cells.

Regardless of the above uncertainties, this is the first preclinical study that combines signal transduction inhibition with adoptive T-cell immunotherapy by targeting BTK and CD19, respectively. Our studies showing that exposure to MCL cells led to a marked increase in inhibitory receptors (“免疫 checkpoint molecules”) on CTL019 that was partially abrogated by cotreatment with ibrutinib. These observations may suggest that this two-pronged antitumor approach derives additional synergy from ibrutinib-mediated T-cell mobilization and from ibrutinib-mediated reduction in inhibitory receptor expression on CAR T cells.

Disclosure of Potential Conflicts of Interest

M.V. Maus and M. Kalos have ownership interest (including patents) in Novartis. S.F. Lacey, M. Milone, C.H. June, S. Gill and M.A. Wasik report receiving commercial research grants from Novartis. S.J. Schuster is a consultant/advisory board member for Pharmacycics, and reports receiving commercial research support from Janssen, Novartis, and Pharmacycics. M. Ruella, S.S. Kenderian, J.A. Fraietta, M.V. Maus, M. Milone, C.H. June, S. Gill, and M.A. Wasik are listed as co-inventors on patents in the area of CAR T cells that are owned by the University of Pennsylvania and licensed to Novartis. All authors work under a research alliance involving the University of Pennsylvania and Novartis pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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