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$BTK^{Cys481Ser}$ drives ibrutinib resistance via ERK1/2, and protects $BTK^{Wild\text{-}Type}$ MYD88 mutated cells by a paracrine mechanism.

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KEYPOINTS

- BTK^{Cys481} mutation results in ERK1/2 mediated survival signaling and ibrutinib resistance in MYD88 mutated cells.
- BTK^{Cys481} mutation confers a protective effect against ibrutinib on neighboring BTK wild-type cells through a paracrine mechanism.

ABSTRACT

Acquired ibrutinib resistance due to BTK^{Cys481} mutations occurs in B-cell malignancies, including those with MYD88 mutations. BTK^{Cys481} mutations are usually sub-clonal, and their relevance to clinical progression remains unclear. Moreover, the signaling pathways that promote ibrutinib resistance remain to be clarified. We therefore engineered $BTK^{Cys481Ser}$ and BTK^{WT} expressing MYD88 mutated WM and ABC DLBCL cells, and observed re-activation of BTK–PLCγ2–ERK1/2 signaling in the presence of ibrutinib in only the former. Use of ERK1/2 inhibitors triggered apoptosis in $BTK^{Cys481Ser}$ expressing cells, and showed synergistic cytotoxicity with ibrutinib. ERK1/2 re-activation in ibrutinib treated $BTK^{Cys481Ser}$ cells was accompanied by release of many pro-survival and inflammatory cytokines, including IL-6 and IL-10 that were also blocked by ERK1/2 inhibition. To clarify if cytokine release by ibrutinib treated $BTK^{Cys481Ser}$ cells could protect BTK^{WT} MYD88 mutated malignant cells, we used a Transwell™ co-culture system, and showed that non-transduced BTK^{WT} MYD88 mutated WM or ABC DLBCL cells were rescued from ibrutinib induced killing when co-cultured with $BTK^{Cys481Ser}$ but not their BTK^{WT} expressing counterparts. Use of IL-6 and/or IL-10 blocking antibodies abolished the protective effect conferred on non-transduced BTK^{WT} by co-culture with $BTK^{Cys481Ser}$ expressing WM or ABC DLBCL cell counterparts. Rebound of IL-6 and IL-10 serum levels also accompanied disease progression in WM patients with acquired BTK^{Cys481} mutations. Our findings show that the $BTK^{Cys481Ser}$ mutation drives ibrutinib resistance in MYD88 mutated WM and ABC DLBCL cells through re-activation of ERK1/2 activation, and can confer a protective effect on BTK^{WT} cells through a paracrine mechanism.

INTRODUCTION

Bruton tyrosine kinase (BTK) is a non-receptor tyrosine kinase that can mediate pro-survival signaling in MYD88 mutated WM and ABC DLBCL cells.[1,2] Activating mutations in MYD88 can also transactivate hematopoietic cell kinase (HCK) in WM and ABC DLBCL cells, which can trigger pro-survival signaling cascades that include BTK, as well as PI3K/AKT and MAPK/ERK1/2.[3] Ibrutinib targets both BTK and HCK, and has demonstrated remarkable clinical activity in MYD88 mutated WM and ABC DLBCL patients.[2,4] Mutated MYD88 may also trigger BCR signaling through activation of SYK.[5] Acquired resistance to ibrutinib is increasingly being recognized with prolonged therapy in chronic lymphocytic leukemia (CLL),[6,7] mantle cell lymphoma (MCL),[8] and WM patients[9] due to somatic mutations affecting BTK^{Cys481} that abrogate BTK-ibrutinib binding. Among these are c.1635G>C and c.1634T>A mutations resulting in a p.Cys481Ser ($BTK^{Cys481Ser}$) that is frequently found in CLL, MCL, and WM patients.[5,7,8] The signaling cascade(s) that contribute to $BTK^{Cys481Ser}$ related ibrutinib resistance remain to be clarified in MYD88 mutated cells. Moreover, in most CLL, MCL, and WM cases, BTK^{Cys481} mutations are sub-clonal and their relevance to clinical disease progression remains unclear.[6,9-11] In this study, we engineered MYD88 mutated WM and ABC DLBCL cells to express $BTK^{Cys481Ser}$ and sought to clarify mechanism(s) that contribute to individual as well as bystander tumor cell resistance to ibrutinib.

MATERIALS and METHODS

Cell Lines and Reagents

$MYD88^{L265P}$ expressing WM (BCWM.1 and MWCL-1) and ABC DLBCL (TMD8, HBL1) cells were used in the studies. WM cell lines were cultured in RPMI 1640 media with 10% FBS, and TMD8 and HBL1 were cultured in IMDM media with 10% and 20% FBS respectively. Media were supplemented with penicillin, streptomycin, and L-glutamine (ThermoFisher). The BTK inhibitor ibrutinib, and ERK1/2 inhibitors ulixertinib (BVD-523) and GDC-0994 were purchased from Selleck Chemicals (Houston, TX). Hyaluronic acid (HA) was purchased from Hyalose, LLC (Austin, TX).

Lentiviral Transduction Experiments

BTK^{WT} or $BTK^{Cys481Ser}$ expressing or control lentiviral vectors were transduced into MYD88 mutated WM and ABC DLBCL cells as previously described.[1,3] The coding sequences for BTK^{WT} or $BTK^{Cys481Ser}$ were cloned into a lentiviral expression vector, pLVX-EF1α-IRES-Puro vector (Clontech Laboratories, Mountain View CA), and expression confirmed by Sanger sequencing as before.[9] Following lentiviral transduction, stable cell lines were selected by culture with 0.5~1.0 ug/ml puromycin. Protein expression levels for BTK^{WT} or $BTK^{Cys481Ser}$ transduced cells were confirmed by immunoblotting for BTK. BTK^{WT} or $BTK^{Cys481Ser}$ expressing WM and ABC DLBCL cell lines were transduced to also express GFP for mixed co-culture study using pLenti-II-CMV-Luc-IRES-GFP lentiviral vector (Applied Biological Materials Inc., Richmond, BC, Canada).

Signaling Studies

Immunoblotting for BTK and its downstream proteins was performed using antibodies for BTK-pY223 (Abcam, Cambridge MA); BTK (Santa Cruz Biotechnology, Dallas TX); PLCγ2-pY759, PLCγ2, ERK1/2-pT202/pY204, ERK1/2, p90RSK-pT359, p90RSK, AKT-pT473, AKT, IkBα-pS32, IkBα (Cell Signaling Technologies, Danvers MA). GAPDH antibody (Santa Cruz Biotechnology) was used for loading control. BTK^{WT} or $BTK^{Cys481Ser}$ expressing WM and ABC DLBCL cells were treated for 2 hours with either DMSO, or 0.1 to 0.5 μM of ibrutinib, ulixertinib, GDC-0994, or a combination of either ibrutinib with ulixertinib or GDC-0994 prior to immunoblotting.

Cytotoxicity Studies

The CellTiter-Glo® Luminescent cell viability assay (Promega, Madison WI) was used to assess the dose-response of inhibitors alone or in combination.[1,3] Cells were seeded into 384 or 96 well plates with the EL406 Combination Washer Dispenser (BioTek Instruments, Inc.) and inhibitors were injected into culture media with the JANUS Automated Workstation (PerkinElmer Inc., Waltham MA). Cells were incubated with inhibitors for 72 hours at 37ºC. Luminescent measurements to assess cell viability were performed using the 2104 Envision® Multilabel Reader (PerkinElmer Inc.). Drug interactions were assessed by CalcuSyn 2.0 software (Biosoft, Cambridge UK) based on Chou TC.[12]

Cell survival was assessed following the treatment with inhibitors using Annexin V-FITC and propidium iodide (PI) staining with the Apoptosis Detection Kit I (BD Pharmingen, San Jose CA). In co-culture studies of GFP^+ BTK^{WT} or $BTK^{Cys481Ser}$ transduced cells with their non-transduced BTK^{WT} counterpart cells, survival analysis was performed on GFP negative population with Annexin V-APC staining.

Ibrutinib Washout Experiment

Washout experiments were also performed to clarify if differences in survival and cytokine release of IL-6 and IL-10 were related to non-BTK covalent ibrutinib interactions. For these experiments, vector only, BTK^{WT} or $BTK^{Cys481Ser}$ transduced MYD88 mutated WM and ABC DLBCL cells were treated with or without ibrutinib for 6 hours, then washed thrice and media replaced without drug. Cell viabilities were assessed at 72 hours as described above.

Co-culture Experiments

For co-culture experiments, BTK^{WT} or $BTK^{Cys481Ser}$ expressing GFP^+ BCWM.1 or TMD8 cells were cultured with ibrutinib (0.5-1.0 uM) for 6 hours. Non-transduced BTK^{WT} GFP negative BCWM.1 or TMD8 cells were then added to wells, and co-cultured for 24~48 hours in the absence or presence of ibrutinib (0.5-1.0 uM) before performing apoptosis analysis. For Transwell™ co-culture experiments, BTK^{WT} or $BTK^{Cys481Ser}$ expressing BCWM.1 or TMD8 cells were pre-treated with serially diluted ibrutinib in the upper chambers of 0.4 um filtered 96 well plates for 6 hours. Non-transduced BTK^{WT} BCWM.1 or TMD8 cells were then seeded into the lower chambers. Fresh ibrutinib was added to maintain the same concentration as the upper chamber, and Transwells™ were incubated for 72 hours. Non-transduced cells in lower compartment were then assessed for viability using the CellTiter-Glo® assay. For anti-IL6 and/or anti-IL10 blocking experiments, 10 µg/ml of neutralizing antibodies (R&D Systems, Inc.) were added to non-transduced cells in the lower Transwell™ chambers. Since hyaluronic acid (HA) is a major extracellular matrix component and a keystone molecule that supports cytokine effects,[13-16] to mimic the impact of microenvironment on the malignant cell survival, we performed above co-culture experiments in the presence of 10 µg/ml of hyaluronic acid (HA). For cytokine rescue experiments, 10 ng/ml CXCL-13, IL-6, IL-10, IL-12, TNF-α,

TNF-β and 100ng/ml MIP-1α, RANTES (R&D Systems, Inc., Minneapolis, MN) were added to the cell culture with or without 10 µg/ml HA. HA alone had no impact on growth or survival of BCWM.1 and TMD8 cells alone or in the presence of ibrutinib (data not shown).

Multiplex Cytokine and ELISA Assays

BTK^{WT} or $BTK^{Cys481Ser}$ expressing BCWM.1 or TMD8 cells were treated with vehicle control, ibrutinib (0.02-0.25 uM), ulixertinib (0.5 uM), or in combination for 36 hours at 37ºC before the supernatants were harvested, aliquoted, and stored at -80 ºC. Sample aliquots were thawed on ice before addition to a customized multiplex plate (Invitrogen) containing 21 analytes (APRIL, BAFF, CD27, CXCL13, Eotaxin, IL-10, IL-12p40, IL-17AF, IL-1RA, IL-2R, IL-4, IL-6, MCP-1, MIF, MIP-1a, MIP-1b, MIP-3a, SDF-1a, TNF-a, TNF-b, sRANKL). ELISA was separately performed for RANTES (R&D Systems). For determination of IL-6 and IL-10 cytokine levels in WM patient sera and cell culture supernatants following ibrutinib washout experiments, ELISA assays (R&D Systems) were performed according to manufacturer's instructions. Sera were stored at -80°C until analysis. The multiplex plate was read on a Bio-Rad Bio-Plex 3D system, and samples were analyzed using xPONENT software. ELISA plates were read on a SpectraMaxM3 Plate Reader (Molecular Devices, Sunnyvale, CA).

Patient Samples

Sera were obtained from relapsed/refractory WM patients who received single agent ibrutinib, and for whom tumor BTK mutation status was established at final serum sampling as previously reported. Three patients in these studies were identified as having BTK^{Cys481} mutations at time of progression, and 6 randomly chosen patients who remained in response were established as having BTK^{WT} disease following last serum sampling. Use of patient samples was approved by the DF/HCC Institutional Review Board.

Quantitative Polymerase Chain Reaction

Total RNA was isolated using AllPrep DNA/RNA Mini Kit (QIAGEN), and cDNA synthesized by SuperScript® III First-Strand Synthesis SuperMix (Life Technologies). Quantitative detection of

mRNA levels for IL-6 and IL-10 was performed using TaqMan® Gene Expression Assays with TaqMan® Gene Expression Master Mix per manufacturer's instructions using an ABI Prism 7500 Sequence Detection System (Applied Biosystems).

Statistical analysis

The statistical significance of differences was analyzed using One-way ANOVA with Tukey's multiple comparisons test by Prism software. Differences were considered significant when $p \leq 0.05$.

RESULTS

$BTK^{Cys481Ser}$ mutation promotes ibrutinib resistance and persistent activation of BTK–PLCγ2–ERK1/2 signaling in MYD88 mutated WM and ABC DLBCL cells.

In our previous work, we identified $BTK^{Cys481Ser}$ as the most common BTK^{Cys481} mutation in MYD88 mutated WM patients with acquired ibrutinib resistance.[9] To explore the functional impact of the $BTK^{Cys481Ser}$ mutation on ibrutinib activity, MYD88 mutated WM and ABC DLBCL cells were transduced with either lentiviral vector alone, or lentiviral vectors expressing BTK^{WT} or $BTK^{Cys481Ser}$. The transduction of $BTK^{Cys481Ser}$ promoted ibrutinib resistance in all MYD88 mutated WM and ABC DLBCL cells with a 1~3 log-fold increase in EC_{50} versus vector only or BTK^{WT} transduced cells (**Figure 1A**). Similar protein expression levels for BTK^{WT} or $BTK^{Cys481Ser}$ were confirmed by immunoblotting in all MYD88 mutated cells (**Figure 1B**). Furthermore, to clarify that the protective effects of $BTK^{Cys481Ser}$ mutation were likely not a consequence of off-target drug interactions, we performed washout experiments. These studies more pronounced protective effects in $BTK^{Cys481Ser}$ versus BTK^{WT} or vector only expressing MYD88 mutated WM and ABC DLBCL cells when compared to findings with continuous ibrutinib treatment supporting that loss of on target binding likely accounted for differences in survival (**Figure 1C**). In contrast to vector only or BTK^{WT} transduced MYD88 mutated cells, all $BTK^{Cys481Ser}$ expressing MYD88 mutated cells showed persistent activation of PLCγ2, which is immediately downstream of BTK, as well as ERK1/2 and its immediate downstream kinase, p90RSK following treatment with ibrutinib (**Figure 1D**). Whereas BTK, PLCγ2

and ERK1/2 remained activated in $BTK^{Cys481Ser}$ expressing cells, IkBa, AKT and p38-MAPK were similarly inhibited in vector only, BTK^{WT} and $BTK^{Cys481Ser}$ expressing cells following ibrutinib treatment (data not shown). Total protein levels for these proteins remained unchanged, and GADPH levels confirmed similar protein loading.

ERK1/2 activation promotes pro-survival signaling in ibrutinib resistant MYD88 mutated WM and ABC DLBCL cells.

Given the persistent activation of ERK1/2 in $BTK^{Cys481Ser}$ expressing MYD88 mutated WM and ABC DLBCL cells following ibrutinib treatment, we next sought to determine the importance of ERK1/2 in mediating ibrutinib resistance. We used two highly selective ERK1/2 inhibitors (ulixertinib, GDC-0994), both under active clinical investigation, to clarify the importance of ERK1/2 signaling in mediating ibrutinib resistance in $BTK^{Cys481Ser}$ expressing cells. Ulixertinib is an allosteric inhibitor, and prompts hyper-phosphorylation of ERK1/2 while blocking its activity.[17] As such, phospho-p90RSK, an immediate downstream kinase of ERK1/2 is used to assess ERK1/2 activity (**Figure 2A, 2B**). Following ulixertinib or GDC-0994 treatment, phospho-p90RSK was reduced in non-transduced, as well as vector only, BTK^{WT}, and $BTK^{Cys481Ser}$ expressing BCWM.1 WM and TMD8 ABC DLBCL cells. More robust reduction in ERK1/2 was also observed in non-transduced, as well as vector only, BTK^{WT}, and $BTK^{Cys481Ser}$ expressing BCWM.1 WM and TMD8 ABC DLBCL cells treated with ulixertinib or GDC-0994 and ibrutinib. These findings show that combining an ERK inhibitor with ibrutinib overcomes ERK1/2 re-activation in the presence of ibrutinib, and that such suppression (as confirmed by downstream p-p90RSK) is greater with ibrutinib and ERK inhibitors combined, even in cell bearing the BTKCys481 mutation (**Figure 2A, 2B**). Importantly, BCWM.1 WM and TMD8 ABC DLBCL cells treated with the combination of ulixertinib with ibrutinib showed higher levels of apoptosis in $BTK^{Cys481Ser}$ expressing BCWM.1 WM and TMD8 ABC DLBCL cells versus either drug alone, with similar levels of apoptosis to their BTK^{WT} counterpart cells (**Figure 2C**). Moreover, the combination of ulixertinib with ibrutinib produced higher levels of tumor cell killing versus either agent alone in both BTK^{WT} and $BTK^{Cys481Ser}$ expressing BCWM.1 WM and TMD8 ABC DLBCL cells by CellTiter-Glo® assay, and showed synergistic interactions with combination index (CI) values below 1.0 at most pharmacologically relevant concentrations (**Figure 2D**). Similar findings were also

observed with GDC-0994 (data not shown). These findings demonstrate that inhibition of ERK1/2 can overcome ibrutinib resistance in MYD88 mutated WM and ABC DLBCL cells.

BTK^{Cys481Ser} expressing WM and ABC DLBCL cells provide a protective effect to BTK^{WT} malignant cells through paracrine mediated pro-survival signaling.

Acquired BTK^{Cys481} mutations following ibrutinib have been reported in CLL, MCL and WM, however in most cases the mutation burden remains low despite clinical disease progression.[6,9,10] To understand how BTK^{Cys481} mutations might impact neighboring BTK^{WT} malignant cells, we first performed mixed co-culture experiments using non-transduced GFP^{-} BCWM.1 WM or TMD8 ABC DLBCL cells with their GFP^{+} BTK^{WT} or BTK^{C481S} cells models. While under Ibrutinib treatment, non-transduced GFP^{-} BCWM.1 or TMD8 cells that were co-cultured with their respective GFP^{+} BTK^{Cys481Ser} expressing showed decreased apoptosis, versus co-culture with their BTK^{WT} counterparts (**Figure 3A, 3B**). Given the protective effect that BTK^{Cys481Ser} expressing cells provide to non-transduced BTK^{WT} MYD88 mutated malignant cells, we next sought to clarify if such a protective effect was mediated by direct cell-cell contact or through a paracrine mechanism without direct cell-cell contact. To address this question, we used a Transwell^{TM} co-culture system that separated non-transduced BTK^{WT} BCWM.1 or TMD8 cells from direct contact with their corresponding vector only, BTK^{WT} or BTK^{Cys481Ser} transduced counterparts in the presence of ibrutinib. These experiments showed that BTK^{Cys481Ser} expressing cells conferred a protective effect on their counterpart non-transduced BTK^{WT} cells versus vector only, or BTK^{WT} transduced cells with a ~1 log fold increase in EC_{50} (**Figure 3C**). The findings support that BTK^{Cys481Ser} expressing MYD88 mutated WM and ABC DLBCL cells can protect their BTK^{WT} counterparts against ibrutinib through a paracrine mechanism.

ERK1/2 activation in BTK^{Cys481Ser} expressing MYD88 mutated cells triggers pro-survival and inflammatory cytokine release in ibrutinib treated cells.

Since the above studies showed that BTK^{Cys481Ser} expressing cells provided a protective effect on neighboring BTK^{WT} malignant cells through a paracrine mechanism, we next sought to identify cytokine(s) that could possibly afford this protective effect. Moreover, since ERK1/2 remains activated

in $BTK^{Cys481Ser}$ expressing cells under ibrutinib treatment, and ERK1/2 is known to trigger pro-survival and inflammatory cytokine release,[18-20] we also sought to delineate its role in paracrine mediated ibrutinib resistance. We performed a multiplex assay to detect cytokines released by either BTK^{WT} or $BTK^{Cys481Ser}$ expressing BCWM.1 WM or TMD8 ABC DLBCL cells following treatment with vehicle control, ibrutinib or ulixertinib alone, or in combination. Cytokine release following drug treatment for 36 hours is shown in **Figure 4.** Higher levels of cytokines, many with a pro-survival and/or inflammatory role, were observed in ibrutinib treated $BTK^{Cys481Ser}$ versus BTK^{WT} expressing cells, and included CXCL-13, IL-6, IL-10, IL-12, MIP-1α, MIP-3α, RANTES, TNF-α, TNF-β (in BCWM.1 WM cells), and CXCL-13, IL-6, IL-10, IL-12, MIP-1α, MIP-1β, TNF-α, TNF-β (in TMD8 ABC DLBCL) cells (**Figure 4**). Importantly, the release for most of these cytokines was reduced by ulixertinib, particularly in combination with ibrutinib. The absolute levels of cytokines produced by BTK^{WT} or $BTK^{Cys481Ser}$ expressing cells following vehicle control or drug treatment are shown in **Supplemental Figure 1**.

IL-6 and/or IL-10 production by $BTK^{Cys481Ser}$ expressing cells confer a protective effect on BTK^{WT} MYD88 mutated WM and ABC DLBCL cells treated with ibrutinib.

To delineate the cytokine(s) released by $BTK^{Cys481Ser}$ cells that protected BTK^{WT} against ibrutinib, we evaluated those cytokines that showed a significant reduction following ibrutinib in BTK^{WT} but not $BTK^{Cys481Ser}$ expressing cells. CXCL-13, IL-6, IL-10, IL-12, MIP-1α, RANTES, TNF-α, and TNF-β were therefore evaluated on ibrutinib treated non-transduced BTK^{WT} BCWM.1 and TMD8 cells. To mimic the impact of microenvironment on the malignant cell survival, we performed the experiments with or without 10 μg/ml hyaluronic acid (HA), a major extracellular matrix component and a keystone molecule that supports cytokine effects.[13-16] HA itself had no impact on growth or survival of BCWM.1 and TMD8 cells alone or in the presence of ibrutinib, but enhanced IL-6 protective effects in BCWM.1 cells (data not shown). In BCWM.1 cells, the addition of IL-6 or IL-10 protected against ibrutinib related cytotoxicity with about 4 fold increase in EC_{50}; MIP-1α, RANTES, TNF-α also showed a protective effect against ibrutinib, with more moderate shifts in EC_{50} following ibrutinib treatment (**Figure 5A)**. In TMD8 cells, IL-10 provided a highly robust rescue effect against ibrutinib related cytotoxicity, with EC_{50} increased by 300 fold. Relative to IL-10, IL-6 provided a more modest (i.e. 5-fold) increase in EC_{50} against ibrutinib in TMD8 cells (**Figure 5B)**. Lastly, both IL-6 and IL-10 were decreased in washout studies with ibrutinib in BTK^{WT} but not $BTK^{Cys481Ser}$ expressing BCWM.1 and

TMD8 cells informing that such differences were likely not a consequence of off-target drug interactions (**Supplemental Figure 2**).

To further clarify whether the protective effect on non-transduced BTK^{WT} malignant cells by $BTK^{Cys481Ser}$ expressing cells was due to their release of IL-6 and/or IL-10, we next performed cytokine blocking experiments. Since a rescue effect was similarly prompted by IL-6 and IL-10 in BCWM.1 WM cells, and robustly by IL-10 alone in TMD8 cells, anti-IL-6 and anti-IL-10 blocking antibodies were added to Transwell^{TM} co-culture with non-transduced BTK^{WT} BCWM.1/$BTK^{Cys481Ser}$ BCWM.1 cells pair, and only anti-IL-10 blocking antibodies to Transwell^{TM} co-culture with non-transduced BTK^{WT} TMD8/$BTK^{Cys481Ser}$ TMD8 cells pair. Importantly, the addition of IL-6 and IL-10 blocking antibodies to Transwell^{TM} co-cultures with non-transduced BTK^{WT} BCWM.1/$BTK^{Cys481Ser}$ BCWM.1 cells pair, and anti-IL-10 blocking antibodies with non-transduced BTK^{WT} TMD8/$BTK^{Cys481Ser}$ TMD8 cells pair abrogated the rescue effects conferred by $BTK^{Cys481Ser}$ cells (**Figure 5C, 5D)**.

Increased IL-6 and IL-10 serum levels accompanies disease progression on ibrutinib in WM patients with acquired $BTK^{Cys481Ser}$ mutations.

Serial serum samples obtained from relapsed/refractory WM patients who received single agent ibrutinib, and for whom tumor BTK mutation status was established at final serum sampling were evaluated for IL-6 and IL-10. Their clinical course and methods for determining their BTK mutation status was previously reported. Three patients in these studies were identified as having BTK^{Cys481} mutations at time of progression, and 6 randomly chosen patients who remained in response were established as having BTK^{WT} disease following last serum sampling. All patients achieved a major response as their best response. The findings from these studies showed that IL-6 and IL-10 levels declined in all patients at time of response, and rebounded at time of progression in those patients in whom BTK^{Cys481} mutations were detected. Among patients who continued to respond, IL-6 and IL-10 serum levels remained decreased (**Figures 6A, 6B**).

IL-6 and IL-10 are triggered by sustained ERK1/2 activation in ibrutinib treated $BTK^{Cys481Ser}$ expressing cells, and can be abrogated by ERK1/2 inhibitors.

To delineate if the persistent production of IL-6 and IL-10 by $BTK^{Cys481Ser}$ expressing cells under ibrutinib treatment is due to sustained ERK1/2 activation, we treated BTK^{WT} and $BTK^{Cys481Ser}$ expressing BCWM.1 WM or TMD8 ABC DLBCL cells with ibrutinib, ulixertinib, or GDC-0994 alone or in combination, and assessed IL-6 and IL-10 mRNA expression. Compared to their BTK^{WT} transduced counterparts, $BTK^{Cys481Ser}$ expressing cells showed significantly higher levels of IL-6 and IL-10 transcription under treatment with ibrutinib (**Figure 7**). Moreover, IL-6 and IL-10 transcription were both markedly reduced in BCWM.1 WM and TMD8 ABC DLBCL cells following treatment with the ERK1/2 inhibitors ulixertinib or GDC-0994 (**Figure 7**).

DISCUSSION

In this study, we sought to delineate the pathway(s) responsible for acquired ibrutinib resistance related to BTK^{Cys481} mutations in MYD88 mutated B-cell malignancies. BTK^{Cys481} mutations that abolish BTK-ibrutinib binding are the most common acquired mutations observed in CLL, MCL and WM patients progressing on ibrutinib, particularly the $BTK^{Cys481Ser}$ somatic variant.[7-9] BTK^{Cys481} mutations may be detected up to a year before clinical progression,[9,11,21] and multiple BTK^{Cys481} mutations can occur within individual patients.[9,21] However, in most progressing patients, BTK^{Cys481} variants are found in a minority of tumor clones,[9,10,22] raising the possibility that non-BTK^{Cys481} mutations also co-exist that contribute to disease progression, or that BTK^{Cys481} mutated cells are able to confer resistance to non-BTK mutated malignant cells. While the former continues to be sought, few co-incidental mutations to BTK^{Cys481} such as PLCγ2 and CARD11 have been identified by targeted and whole exome sequencing. For this reason, we sought to clarify if BTK mutated cells could promote resistance of neighboring non-BTK mutated malignant cells.

Our findings showed that expression of the $BTK^{Cys481Ser}$ mutation resulted in uniform loss of BTK pathway suppression in the presence of ibrutinib, with activation of downstream PLCγ2 signaling observed in all MYD88 mutated WM and ABC DLBCL cells. The introduction of BTK^{Cys481} showed higher levels of resistance to ibrutinib in ABC DLBCL versus WM cells, and may reflect differences in non-covalent interactions with other pro-survival targets such as HCK.[3] Importantly, sustained activation of ERK1/2 along with its immediate downstream kinase, p90RSK, uniformly occurred in all BTK^{Cys481} mutated WM and ABC DLBCL despite ibrutinib treatment. In contrast, an ibrutinib

suppressive effect on ERK1/2 signaling was present in ibrutinib treated WM and ABC DLBCL cells transduced with vector only or BTK^{WT}. While both ERK1/2 and AKT are known to be transactivated by BTK,[23,24] only sustained activation of ERK1/2 following ibrutinib treatment was observed in $BTK^{Cys481Ser}$ MYD88 mutated WM and ABC DLBCL cells. The importance of sustained ERK1/2 signaling to ibrutinib resistance in MYD88 mutated WM and ABC DLBCL cells was demonstrated by co-treatment of $BTK^{Cys481Ser}$ expressing cells with ibrutinib and selective ERK1/2 inhibitors. The combined use of an ERK1/2 inhibitor with ibrutinib resulted in synergistic tumor cell killing in $BTK^{Cys481Ser}$ expressing cells versus either agent alone. The findings could reflect a continued role for ibrutinib against BTK^{Cys481} mutated cells, since ibrutinib is known to suppress the activity of other kinases such as HCK that support growth and survival signaling in MYD88 mutated cells.[3] While these studies, to our knowledge, provide the first experimental evidence that sustained ERK1/2 activation accompanies mutated BTK^{Cys481} signaling in ibrutinib resistant cells, the re-activation of ERK1/2 was also observed in serially obtained CLL cells from a patient that initially responded to, and subsequently progressed on ibrutinib after acquisition of a BTK^{Cys481} mutation.[25] Therefore, the relevance of ERK1/2 activation following acquisition of a BTK^{Cys481} mutation may apply to non-MYD88 mutated B-cell diseases, and warrant further studies. Other treatment options including targeting BCL2 with venetoclax, and use of non-covalent inhibitors of BTK that rely on binding to non-BTK^{Cys481} targets such as vecabrutinib (SNS-062) represent other strategies for overcoming BTK^{Cys481} dependent ibrutinib resistance and are under investigation. A clinical trial examining venetoclax (NCT02677324) in previously treated WM patients, including those that progressed on ibrutinib is underway in WM patients. A clinical trial with vecabrutinib in B-lymphoid malignancies is also underway (NCT03037645).

A novel finding from these studies was the recognition that BTK^{Cys481} mutated cells could bestow a protective effect on BTK^{WT} cells. BTK^{Cys481} are typically sub-clonal, and these findings provide an explanation for how sub-clonal BTK mutated cells could promote widespread resistance against ibrutinib. A paracrine-mediated mechanism was surmised from results showing that a protective effect was conferred by both direct as well as indirect (via Transwell^{TM}) co-culture of $BTK^{Cys481Ser}$ and BTK^{WT} MYD88 mutated WM and ABC DLBCL cells. Given these findings, we sought to clarify if cytokine(s) prompted the protective effects afforded by BTK^{Cys481} mutated cells. ERK1/2 signaling is triggered by BTK, and is responsible for a wide range of inflammatory and pro-survival cytokine signaling. Using a

customized multiplex cytokine assay system, we evaluated cytokines with a putative pro-survival role in MYD88 mutated B-cell diseases, and those blocked by ibrutinib in patients. The findings herein showed that loss of ibrutinib mediated suppression of many inflammatory and pro-survival cytokines accompanied expression of $BTK^{Cys481Ser}$, and were directly related to sustained ERK1/2 activation. Use of ERK1/2 inhibitors abolished the release of most aberrantly expressed cytokines that we evaluated, as well as the transcription and release of IL-6 and IL-10 that mediated the most impressive pro-survival effects against ibrutinib in MYD88 mutated cells. Both IL-6 and IL-10 are known triggers of strong pro-survival signaling including JAK/STAT in MYD88 mutated WM and ABC DLBCL cells.[26-30] Many of the other ERK1/2 triggered cytokines in $BTK^{Cys481Ser}$ such as RANTES, which themselves showed modest direct pro-survival effects on ibrutinib treated MYD88 mutated cells could act as triggers of microenvironmental IL-6 or IL-10 release.[31] In previous work, we observed that both IL-6 and IL-10 serum levels decreased in WM patients responding to ibrutinib, and further to this work we show in these studies their subsequent rebound in progressing patients with acquired BTK^{Cys481} mutations consistent with the *in vitro* findings presented herein.[32] Other cytokines may also contribute to disease resistance related to BTK^{Cys481} mutations. Ahn et al[21] observed rebound in CCL3 and CCL4 levels after initial decrease in CLL patients with acquired BTK Cys481 mutations that progressed on ibrutinib. Decreased CCL4 but not CCL3 was also observed by us in ibrutinib responding WM patients, though changes in the former were less pronounced versus those observed in IL-6 and IL-10.[32]

Several ERK1/2 inhibitors are currently under clinical investigation,[17,33,34] and could be utilized with ibrutinib in patients with BTK^{Cys481} mutations. Such a strategy would have the benefit of continuing ibrutinib to suppress BTK^{WT} clones and non-BTK kinases such as HCK within BTK^{Cys481} mutated clones that promote MYD88 directed growth and survival, whilst the addition of an ERK1/2 inhibitor would suppress pro-survival signaling triggered by the BTK^{Cys481} mutation, and its paracrine mediated protective effects on BTK^{WT} clones. Conceivably, intervention with an ERK1/2 inhibitor could also occur before clinical progression occurs on ibrutinib, since BTK^{Cys481} mutations can often be detected up to a year beforehand. Studies to address the use of ERK1/2 inhibitors, and optimal timing for drug intervention are needed to address these points. Lastly, the use of IL-6 and/or IL-10 blocking agents is also suggested by these studies as an alternative strategy to overcome ibrutinib resistance in patients with BTK^{Cys481} mutations.

Since PLCγ2 activating mutations are also found in patients with acquired ibrutinib resistance, including those with and without MYD88 mutated disease,[6,9,21,22,35,36] these findings may also be relevant since ERK1/2 activation is downstream of both BTK and PLCγ2. Further work to elucidate the importance of ERK1/2 signaling, and the potential use of ERK1/2 inhibitors to overcome PLCγ2 mutations appears warranted.

In summary, our findings provide experimental evidence for the first time that an acquired BTK^{Cys481} mutation drives ibrutinib resistance in MYD88 mutated WM and ABC DLBCL cells through sustained ERK1/2 activation that can confer a protective effect on BTK^{WT} cells through a paracrine mechanism. Use of ERK1/2 inhibitors along with ibrutinib abolished the protective effects triggered by ERK1/2 activation. The findings provide a framework for investigation of ERK1/2 inhibitors to overcome ibrutinib mediated resistance in MYD88 mutated diseases.

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AUTHOR CONTRIBUTIONS

GY and SPT conceived and designed the experiments, and wrote the manuscript. GY, GGC, and ZRH performed the data analysis; JGC and XL performed transduction experiments, PCR-based studies, and immunoblotting. JGC, GY performed drug treatment and cell viability assessments. JGC, MM performed the multiplex cytokine studies. LX, NT, MGD, MLG, AK prepared samples, and performed genotyping studies. JJC, TD, PS, KM, JG, CPT, and SPT provided patient care, obtained consent and were responsible for sample collection.

DISCLOSURES

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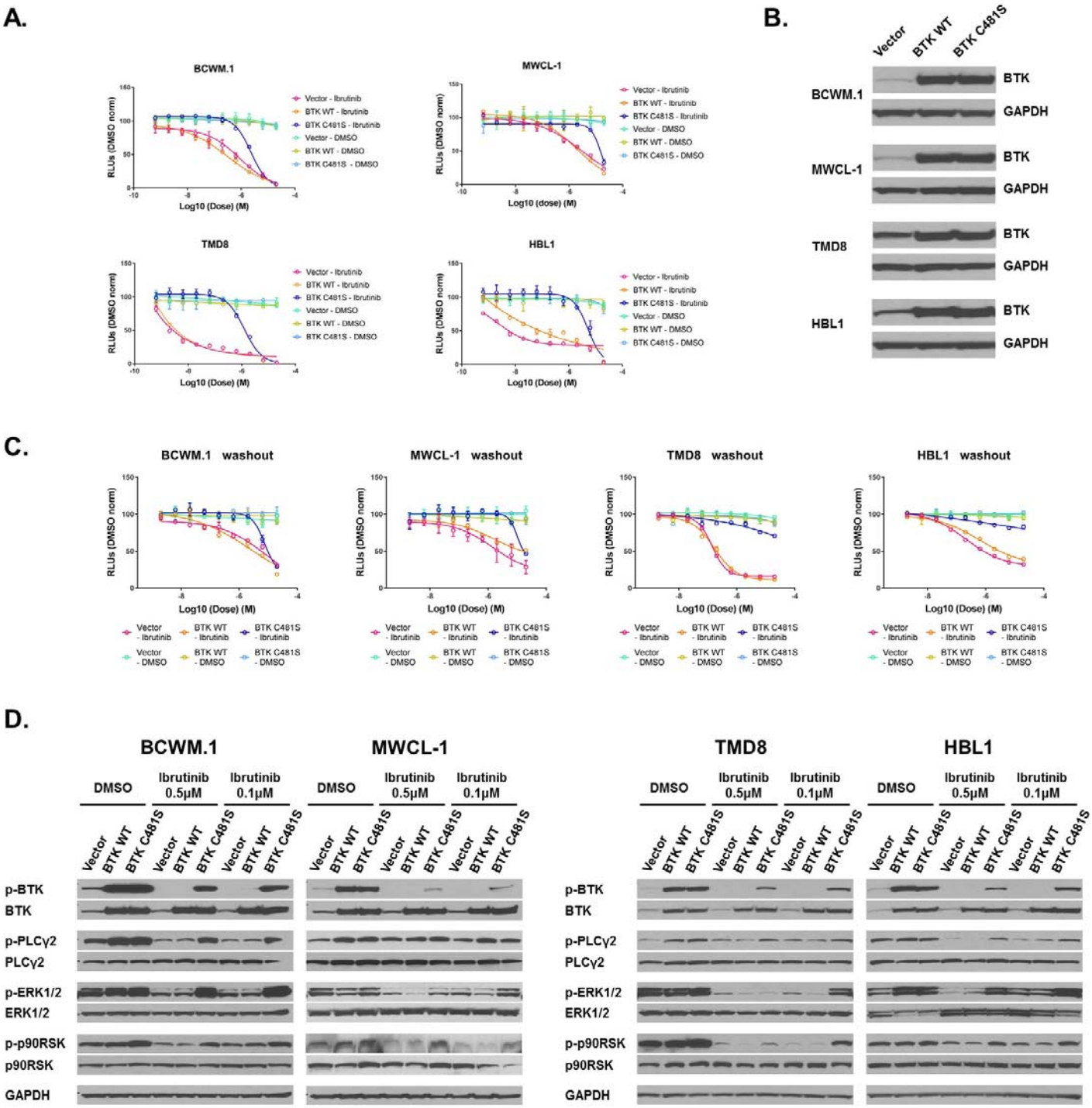

Figure 1. BTK^{Cys481Ser} mutation promotes ibrutinib resistance and persistent activation of BTK and ERK1/2 signaling in MYD88 mutated WM and ABC DLBCL cells. MYD88 mutated WM (BCWM.1, MWCL-1) and ABC DLBCL (TMD8, HBL1) cells were transduced with vector alone, or

vectors expressing either BTK^{WT} or $BTK^{Cys481Ser}$ (BTK^{C481S}). Dose dependent survival determined by CellTiter-Glo® Luminescent cell viability assay for vector only, BTK^{WT} or BTK^{C481S} transduced MYD88 mutated WM and ABC DLBCL cells following treatment with ibrutinib for 72 hours. Each data point was calculated relative to DMSO control. X-axis shows ibrutinib log concentration (**A**). Similar levels of total BTK protein expression were obtained in MYD88 mutated WM and ABC DLBCL cell following transduction with either BTK^{WT} or BTK^{C481S} vectors (**B**). Cell viabilities at 72 hours for vector only, BTK^{WT} or $BTK^{Cys481Ser}$ transduced MYD88 mutated WM and ABC DLBCL cells following treatment with ibrutinib for 6 hours then washout and replacement of media without ibrutinib. Each data point was calculated relative to DMSO control. X-axis shows ibrutinib log concentration (**C**). Immunoblotting studies depicting impact of vector only, BTK^{WT} or BTK^{C481S} expression in MYD88 mutated WM and ABC DLBCL cells on BTK, PLCγ2, ERK1/2 and p90RSK signaling following treatment with vehicle control (DMSO) or ibrutinib (0.5, 0.1 μM) for 2 hours. GAPDH used as protein loading control (**D**).

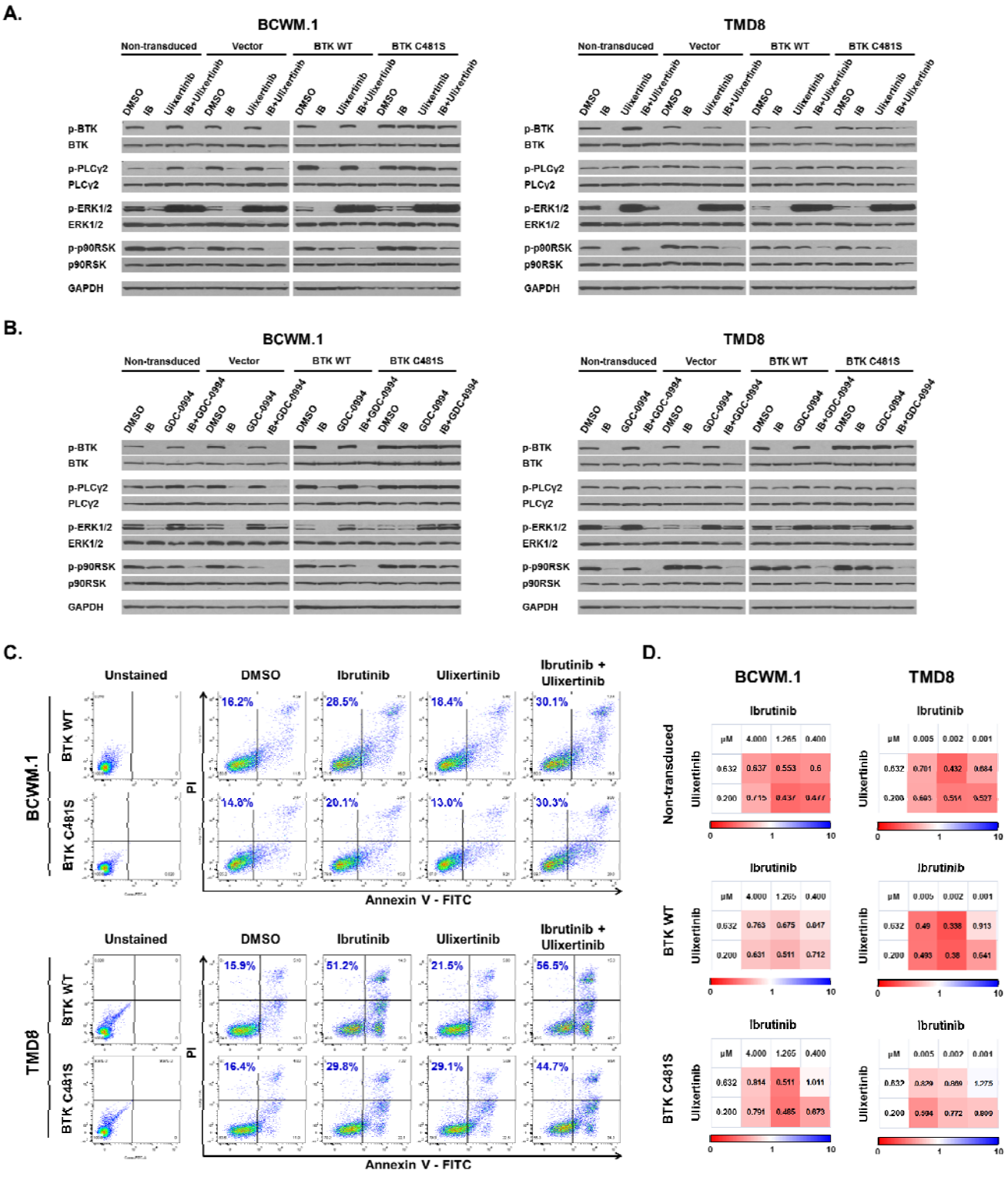

Figure 2. ERK1/2 activation promotes pro-survival signaling in ibrutinib resistant MYD88 mutated WM and ABC DLBCL cells. Immunoblotting studies depicting BTK, PLCγ2, ERK1/2 and

p90RSK signaling in non-transduced, vector only, BTK^{WT} or $BTK^{Cys481Ser}$ (BTK^{C481S}) MYD88 mutated WM (BCWM.1) and ABC DLBCL (TMD8) cells following treatment with vehicle control, ibrutinib, or ERK1/2 inhibitors (ulixerinib, GDC-0994) alone or with ibrutinib for 2 hours. GAPDH used as protein loading control. Data for ulixertinib is shown in Panel **(A)**; and for GDC-0994 in panel (**B)**. Cellular apoptosis determined by Annexin V-FITC and propidium iodide (PI) staining for BTK^{WT} or BTK^{C481S} expressing WM and ABC DLBCL cells following treatment with vehicle control, ibrutinib (1.0μM) or ulixertinib (2.0μM) alone or in combination. Percent of cells staining for both Annexin V and PI is depicted in each panel. Results are representative from studies performed in triplicate **(C)**. Dose dependent survival determined by CellTiter-Glo® Luminescent cell viability assay for vector only, BTK^{WT} or BTK^{C481S} transduced MYD88 mutated WM and ABC DLBCL cells following treatment with ibrutinib and ulixertinib at pharmacologically relevant dosimetry for 72 hours. Synergism was assessed by combination index (CI) analysis, with the heat maps depicting the CI values at varying dosimetry for ibrutinib and ulixertinib. CI values < 1 denote synergistic interactions **(D)**.

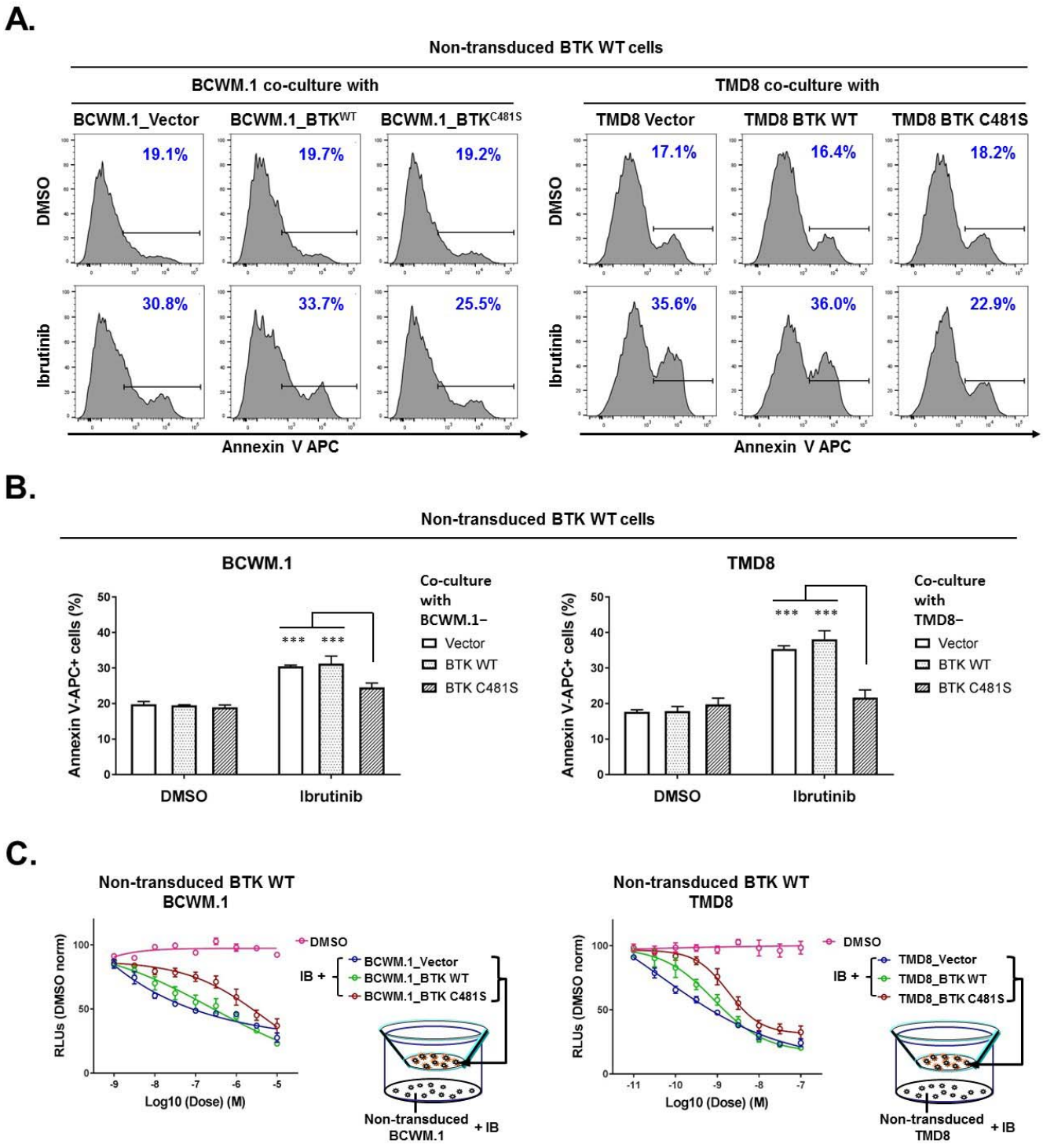

Figure 3. BTK^{Cys481Ser} expressing WM and ABC DLBCL cells confer a protective effect to BTK^{WT} malignant cells through paracrine mediated pro-survival signaling. For co-culture experiments, BTK^{WT} or BTK^{Cys481Ser} expressing GFP^{+} BCWM.1 or TMD8 cells were cultured with ibrutinib (0.5~1.0

μM) for 6 hours. Non-transduced BTK^{WT} GFP^{-} BCWM.1 or TMD8 cells were then added to wells, and co-cultured for 24~48 hours in the absence or presence of ibrutinib (0.5~1.0 μM) Annexin V–APC staining was used to assess apoptotic changes on non-transduced GFP^{-} cells. Experiments were done in triplicates and the representative plots with the percentage of apoptotic cells are shown on panel (**A**). The statistics for all three experiments are displayed in panel (**B**). For Transwell™ co-culture experiments, BTK^{WT} or $BTK^{Cys481Ser}$ expressing BCWM.1 WM or TMD8 ABC DLBCL cells were pre-treated with vehicle control or ibrutinib at indicated concentrations in the upper chambers of 0.4 um filtered plates for 6 hours. Non-transduced counterparts were then cultured for 72 hours with added ibrutinib at the indicated concentrations in the lower compartments, and then assessed for viability using the CellTiter-Glo® assay. The single (DMSO) line shows viability of non-transduced cells with drug vehicle control alone. X-axis shows ibrutinib log concentration (**C**). IB; Ibrutinib.

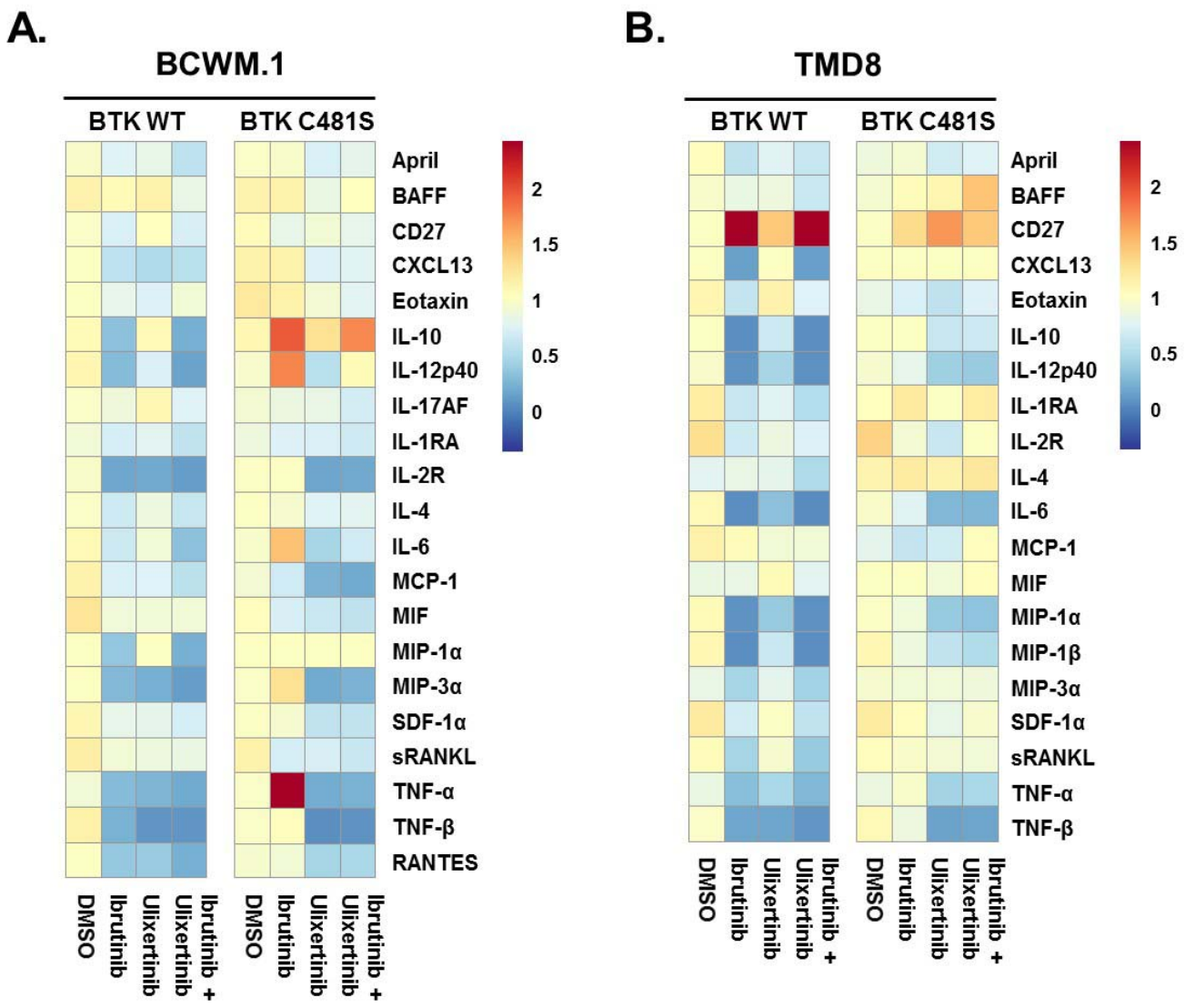

Figure 4. ERK1/2 activation in BTK^{Cys481Ser} expressing MYD88 mutated cells triggers pro-survival and inflammatory cytokine release in ibrutinib treated cells. Cytokine production relative to untreated BTK^{WT} or BTK^{C481S} expressing BCWM.1 WM (**A**) and TMD8 ABC DLBCL (**B**) cells is shown following treatment with either vehicle control (DMSO), ibrutinib, ulixertinib or combination of ibrutinib and ulixertinib. Fold changes are indicated by the color scale, and dark red boxes indicate scales above the highest range (≥2.39).

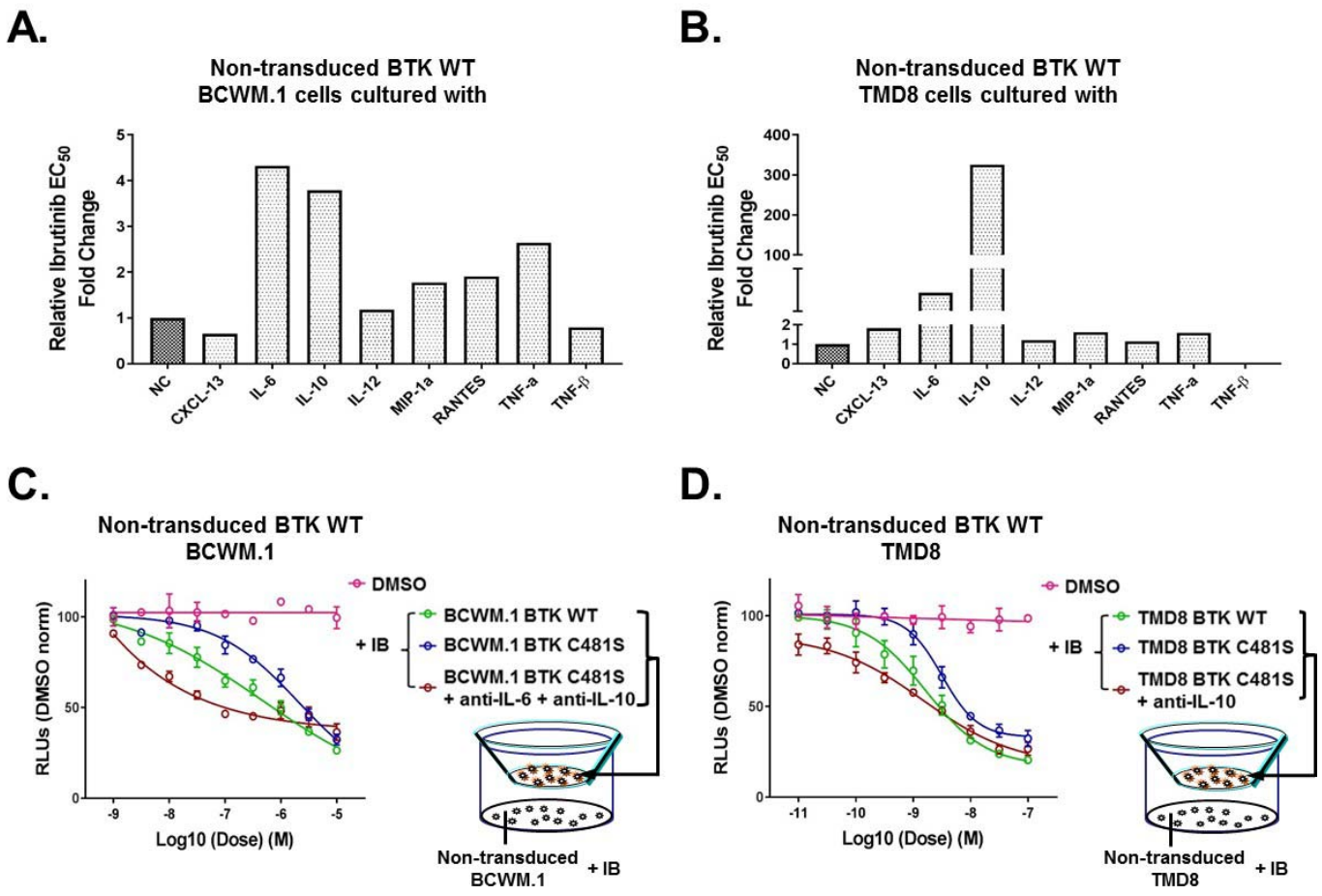

Figure 5. IL-6 and/or IL-10 production by BTK^{Cys481Ser} expressing cells confer a protective effect on BTK^{WT} MYD88 mutated WM and ABC DLBCL cells treated with ibrutinib. Non-transduced BTK^{WT} BCWM.1 **(A)** and TMD8 **(B)** cells were treated with a serial diluted ibrutinib in the presence or absence of cytokines (10 ng/mL CXCL-13, IL-6, IL-10, IL-12, TNF-α, TNF-β and 100ng/ml MIP-1α, RANTES), and the relative fold change to no cytokine control in EC_{50} for ibrutinib is shown. For Transwell^{TM} co-culture experiments, BTK^{WT} or BTK^{Cys481Ser} expressing BCWM.1 **(C)** or TMD8 **(D)** cells were pre-treated with vehicle control or a serial diluted ibrutinib at indicated concentrations in the upper chambers of 0.4 um filtered plates for 6 hours. Non-transduced counterparts were then added to lower chambers with or without blocking antibodies to IL-6 and IL-10 (10 ug/mL) for BCWM.1 WM cells, and to IL-10 alone (10 ug/mL) for TMD8 ABC DLBCL cells. Ibrutinib was then added to the lower chambers to keep the same concentrations and incubated for 72 hours. Viability was assessed by the CellTiter-Glo® assay. IB: Ibrutinib. The single (DMSO) line

shows viability of non-transduced cells with drug vehicle control alone. X-axis shows ibrutinib log concentration.

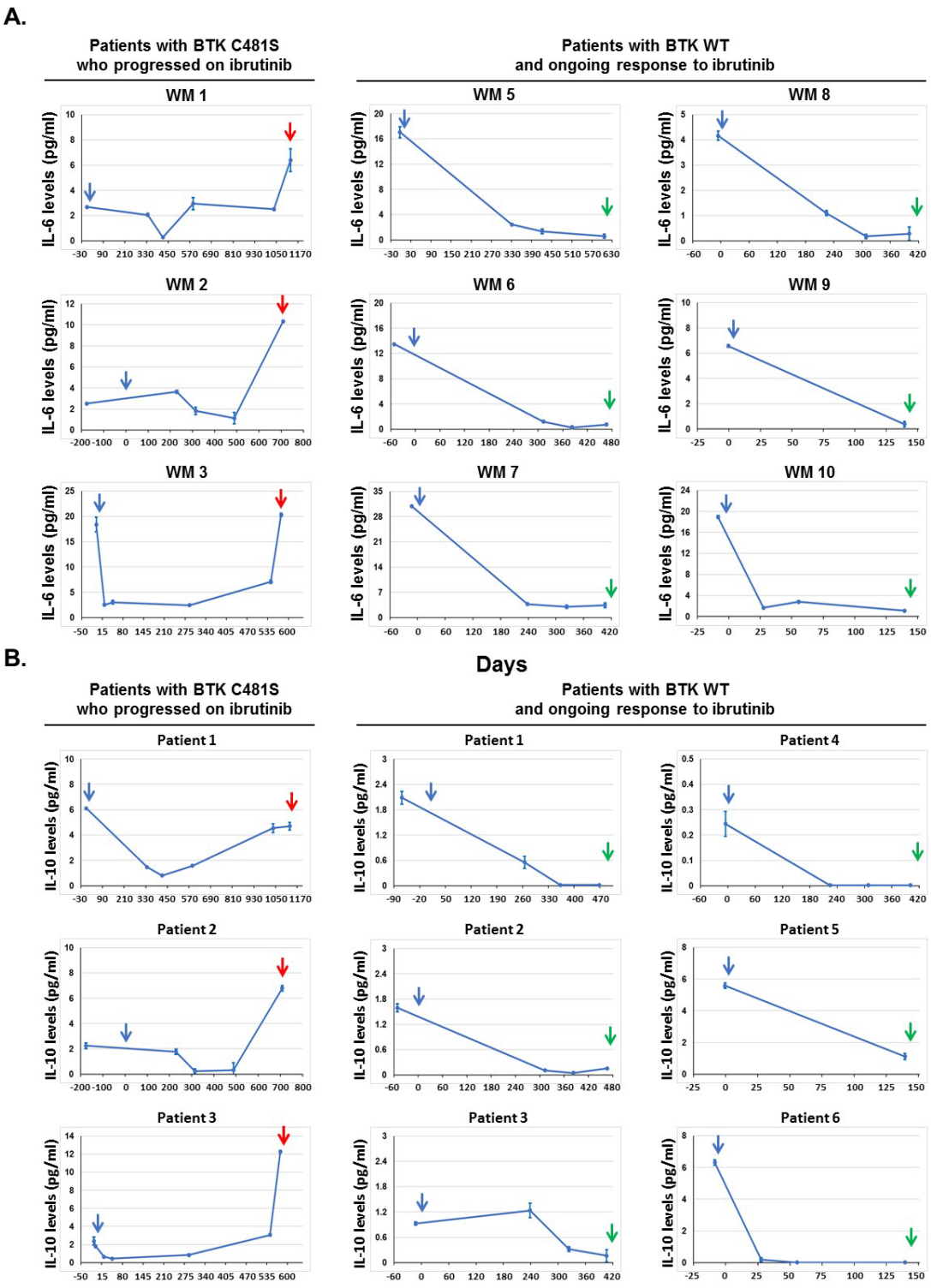

Figure 6. IL-6 and IL-10 cytokine levels in MYD88 mutated WM patient sera following ibrutinib treatment. The IL-6 and IL-10 cytokine levels were detected by ELISA in MYD88 mutated WM

patients with acquired $BTK^{Cys481Ser}$ mutation and progressed on ibrutinib as well as patients with BTK^{WT} and continued response to ibrutinib. Blue arrows indicate ibrutinib start date. Red arrows indicate date at BTK^{Cys481} mutations were first noted in patients with disease progression, while green arrows show determination point for BTK wild-type status for those patients with ongoing response. All patients achieved a major response, and their clinical course and determination of BTK mutation status was previously reported.[8]

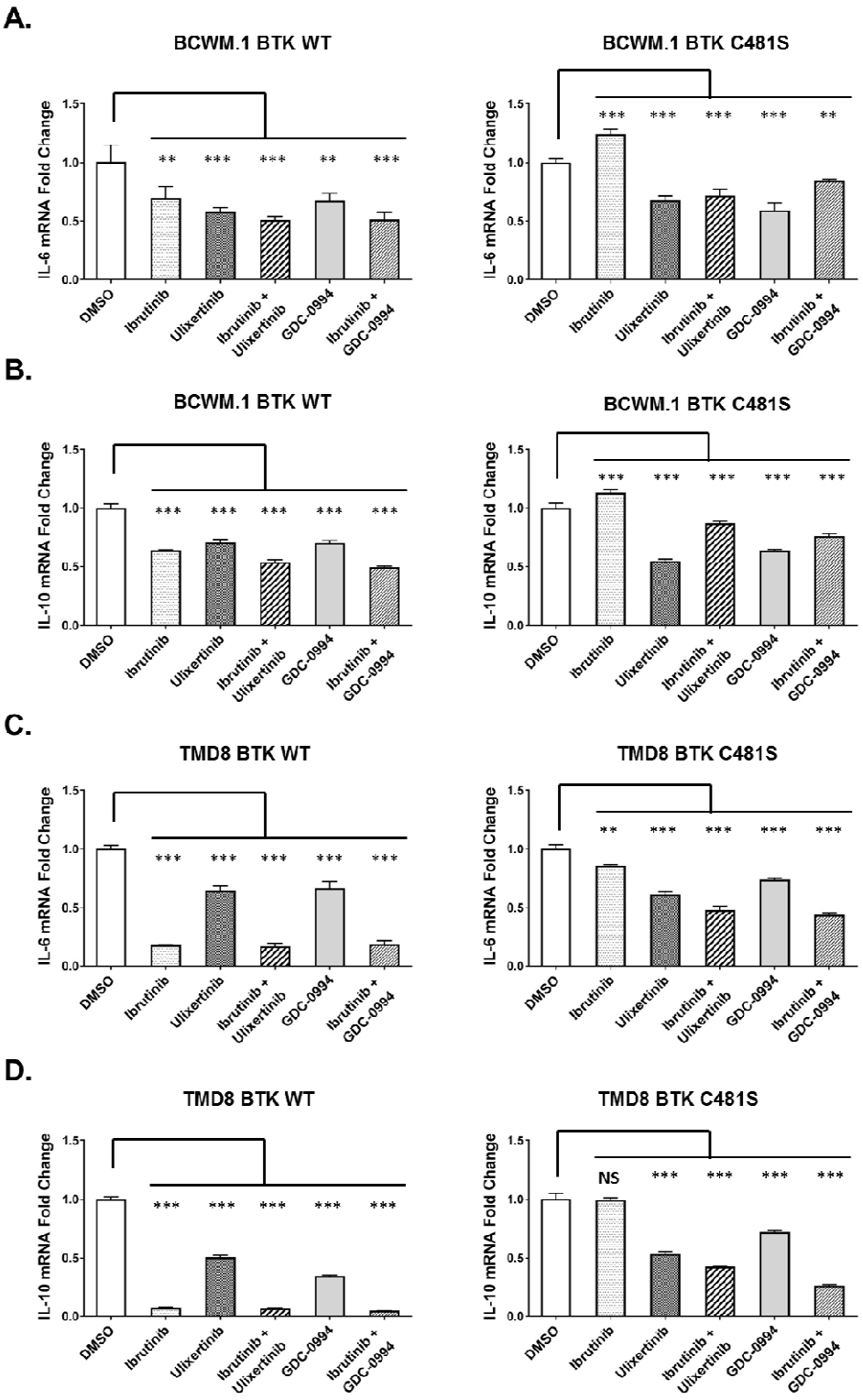

Figure 7. IL-6 and IL-10 are triggered by sustained ERK1/2 activation in ibrutinib treated BTK^{Cys481Ser} expressing cells, and can be abrogated by ERK1/2 inhibitors. BTK^{WT} and

$BTK^{Cys481Ser}$ expressing BCWM.1 WM or TMD8 ABC DLBCL cells were treated with ibrutinib, ulixertinib, or GDC-0994 alone or in combination, and assessed for IL-6 and IL-10 mRNA expression by TaqMan® Gene Expression Assays. $BTK^{Cys481Ser}$ expressing BCWM.1 WM and TMD8 ABC DLBCL cells showed sustained IL-6 **(A, C)** and IL-10 **(B, D)** transcription following ibrutinib treatment that was abrogated by the ERK1/2 inhibitors ulixertinib and GDC-0994.

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$BTK^{Cys481Ser}$ drives ibrutinib resistance via ERK1/2, and protects $BTK^{Wild\text{-}Type}$ MYD88 mutated cells by a paracrine mechanism

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