Loss of TNFAIP3 enhances MYD88L265P-driven signaling in non-Hodgkin lymphoma

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Abstract

MYD88 mutations are one of the most recurrent mutations in hematologic malignancies. However, recent mouse models suggest that MYD88L265P alone may not be sufficient to induce tumor formation. Interplay between MYD88L265P and other genetic events is further supported by the fact that TNFAIP3 (A20) inactivation often accompanies MYD88L265P. However, we are still lacking information about the consequence of MYD88L265P in combination with TNFAIP3 loss in human B cell lymphoma. Review of our genetic data on diffuse large B cell lymphoma (DLBCL) and Waldenstrom macroglobulinemia (WM), found that a large percentage of DLBCL and WM cases that have a MYD88 mutation also harbor a TNFAIP3 loss, 55% DLBCL and 28% of WM, respectively. To mimic this combination of genetic events, we used genomic editing technology to knock out TNFAIP3 in MYD88L265P non-Hodgkin’s lymphoma (NHL) cell lines. Loss of A20 expression resulted in increased NF-κB and p38 activity leading to upregulation of the NF-κB target genes BCL2 and MYC. Furthermore, we detected the increased production of IL-6 and CXCL10 which led to an upregulation of the JAK/STAT pathway. Overall, these results suggest that MYD88L265P signaling can be enhanced by a second genetic alteration in TNFAIP3 and highlights a potential opportunity for therapeutic targeting.

Introduction

Next-generation sequencing data has revealed that MYD88 mutations are one of the most recurrent mutations in hematologic malignancies and are found in 20% of lymphomas (COSMIC data base1). While it is detected in many subtypes of B cell malignancies, its prevalence is highest in Waldenstrom macroglobulinemia (90–100%, WM), primary CNS lymphomas (79%), and the activated B cell subtype of diffuse large B cell lymphoma (39%, ABC-DLBCL)2–4. The most common MYD88 mutation described thus far is a single base pair mismatch resulting in an amino acid switch from lysine to proline at position 265 (MYD88L265P). MYD88 is an adaptor protein which acts downstream of the Toll-like receptor (TLR) and interleukin-1 pathways5. MYD88 activation leads to IRAK1/4 recruitment and further downstream activation and phosphorylation of TRAF6 and TAK1 resulting in NF-κB activation6. MYD88L265P is a constitutively active form of the protein and its expression leads to dysregulated NF-κB and STAT3 signaling2. Additionally, we have shown in a recent study that TAK1 is an essential player in the MYD88L265P pathway and that it contributes to cell proliferation and cytokine secretion7. Together, these data suggest that mutant forms of MYD88 drive development of lymphoma. However, it has recently been shown in mouse models that MYD88L265P alone is not sufficient to induce tumor formation and requires additional genetic
hits, such as loss of the TNFAIP3 tumor suppressor (encodes for the A20 protein) or BCL2 upregulation.8,9

Deletion or mutations in TNFAIP3 on 6q23 are commonly found in DLBCL and WM,10,11 and when combined with a MYD88 mutation may further lead to deregulated NF-kB activation. A20 is an inducible ubiquitin-modifying enzyme and part of the NF-kB-induced negative feedback loop.12 Its role as a tumor suppressor gene in hematological malignancies has been shown in various studies, where restoring of A20 expression in A20 deficient cell lines lead to induction of apoptosis, cell growth arrest, and downregulation of NF-kB target genes.10,13,14 Furthermore, it has been shown that A20 expression is rapidly induced in cells to counteract MYD88-driven proliferation and NF-kB activation.8

The mechanistic interplay and downstream consequence of MYD88L265P in combination with additional genetic hits have not been fully defined in human lymphoma models of MYD88L265P. From a clinical perspective, further insight on MYD88-driven proliferation is important for therapeutic targeting of this pathway. TLR signaling inhibitors have been shown to have an effect on tumor growth in MYD88 mutant cell line models and patient-derived DLBCL tumor xenograft mouse models.15,16 Alternatively, in a recent phase I/II clinical trial in relapsed or refractory ABC-DLBCL it was shown that 80% of patients who harbor a MYD88 together with a CD79B mutation were sensitive to the B cell receptor (BCR) signaling inhibitor ibrutinib. The same study also showed that A20 expression is induced in cells to counteract MYD88-driven proliferation and NF-kB activation.8

Cell lines and establishment of TNFAIP3 knockout clones

The MWCL cell line was established and characterized at Mayo Clinic.22 The BCWM cell line was a kind gift from Dr. Steve Treon and the HBL-1 cell line was kindly provided by Dr. Thomas Witzig. Cell line authentication is described in Supplemental Methods. All cells lines were maintained in RPMI 1640 medium with 10–20% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 µg/ml) were added. Cells were periodically checked for mycoplasma by PCR and were found to be negative. All cell lines were cultivated at 37 °C and 5% CO₂. Transcription activator-like effector nuclease (TALENs) specific for targeting exon 5 of the TNFAIP3 gene were designed by the Mayo Clinic Genetics and Model Systems Core using the FuxX system as previously described.23 Exon 5 was targeted because it is present in all reported TNFAIP3 isoforms. Cells were transfected with 10 µg of each TALEN vector arm by using the AMAXA® nucleofection system (Amaxa, Cologne, Germany). To track successful transfection, cells were cotransfected with a 2 µg GFP expressing plasmid. After 48 h, cells that have incorporated the GFP expression plasmids were isolated by single cell sorting at the Mayo Flow Cytometry Facility. Restriction enzyme digest was used to initially identify clones that carried the frameshift mutation.

Material and methods

Patients, whole-exome sequencing, and copy number analysis

This study was reviewed and approved by the human subjects review board of Mayo Clinic and the University of Iowa, and written informed consent was obtained from all participants. For DLBCL, identification of MYD88 mutant cases was done using whole exome sequencing (WES) data from 145 newly diagnosed DLBCL tumors. WES data from tumor-normal pairs (n = 56)7,18 and tumors embedded in FFPE (n = 89) were combined and analyzed together as described in Supplemental Methods. TNFAIP3 copy number loss was identified using WES (n = 56) or whole genome copy number data (n = 89) from the OncoScan array (Affymetrix, Santa Clara, CA, USA) and analyzed as described in Supplemental Methods. The cell of origin was determined using the Hans algorithm, gene expression profiling, or NanoString technology.19 For WM, identification of MYD88 mutant cases has been described previously using WES or allele-specific PCR (ASO-PCR)7 and TNFAIP3 copy number loss was assessed using real-time quantitative PCR. Briefly, genomic DNA was extracted from 29 WM patients and a TaqMan™ copy number assays probe (Thermo Scientific, Waltham, MA) for TNFAIP3 were used. All qPCR reactions were performed using BioRad CXF96 instrument and the results are expressed as relative units based on calculation 2^ΔΔCt, which gives the relative amount of target gene normalized to the endogenous control. A copy number loss was defined using a cutoff based the mean of the normal controls (n = 5) minus 4 standard deviations, a value of 0.8 was defined as the cutoff for TNFAIP3 loss (Supplemental Figure 1).
Quantitative real-time PCR and RNA-Seq

Total RNA was extracted using the RNeasy Mini Plus Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. cDNA was synthesized using the SuperScript III First-Strand Synthesis kit (Invitrogen, Waltham, MA, USA). Quantitative reverse transcriptase-PCR was performed using either TaqMan probes (Applied Biosystems, Invitrogen, Carlsbad, CA) or probe-based predesigned qPCR primers (IDT, Coralville, IA, USA). All qPCR reactions were performed using BioRad CFX96 instrument. The results are expressed as relative units based on calculation 2^−ΔΔCT, which gives the relative amount of target gene normalized to the endogenous control. For RNAseq, total RNA was extracted using the miRNeasy Mini Plus Kit (Qiagen GmbH, Hilden, Germany). Library preparation and RNA-sequencing were carried out by the Mayo Clinic Genome Analysis Core. Library preparation was done using the Standard TruSeq v2 for mRNA (Illumina, San Diego, CA, USA) and sequencing was carried out on Illumina HiSeq 4000 (Illumina, San Diego, CA, USA). Data analysis was performed by the Mayo Clinic Bioinformatics Core using the Mayo Clinic mRNA-Seq analysis pipeline MAPRSeq (v2.1.1.). A detailed description of the secondary analysis can be found in Supplemental Methods.

Western blot analysis

Cells were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) with protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Protein extracts were clarified by centrifugation, resolved by SDS-PAGE using PROTEAN® TGX™ gels (Bio-Rad Laboratories, Hercules, CA), and transferred to PVDF membranes. Antibodies used in the study are listed in Supplemental Methods.

Cytokine expression analysis

Cells where cultured for 48 h under normal culturing conditions and samples were analyzed using the Human ProcartaPlex™ Simplex Kit for IL-6 and CXCL10 (Invitrogen, Waltham, MA, USA). Samples were run in duplicate and the assay was performed according to the manufacturer’s instructions. Plates were read on a Lumines-200 system (Luminex, Austin, TX, USA) and analyzed using Star Station software (Applied Cytometry, Sheffield, UK).

Proliferation assay

Proliferation assay methods have been described previously. Briefly, cell lines were plated (10 or 25 × 10³ cells/well) in culture media in the presence of either DMSO (Thermo Scientific, Waltham, MA, USA) or ibrutinib (Chemitek, Indianapolis, IN, USA) at the indicated doses for 48 h. After 20 h of incubation, cells were pulsed with 0.05 mCi tritiated thymidine (Amersham, Piscataway, NJ, USA) and ³H-thymidine incorporation levels were determined using a MicroBeta TriLux (PerkinElmer, Waltham, MA, USA). All raw counts were normalized to the DMSO control to set DMSO control to 100.

Statistics

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, USA). p-Values for all experiments have either been calculated using the 2-tailed Student t-test, or if applicable, the Wilcoxon–Mann–Whitney test was used. Fisher’s exact test was used to compare the correlation between genomic events. p-Value of ≤0.05 was considered to be statistically significant. All error bars are shown as standard deviation (SD).

Results

MYD88 and TNFAIP3 genetic alterations in DLBCL and WM

Deubiquitinating enzymes such as A20 counteract E3-ligase activity, inhibit TRAF6 activity, and negatively regulate MYD88-driven TAK1, NF-κB, and p38 activation (Fig. 1a). To better understand the impact of TNFAIP3 loss on MYD88L265P in DLBCL and WM, we first wanted to determine the frequency of MYD88 mutations in combination with TNFAIP3 genetic alterations. Using genetic data from 145 cases of DLBCL, we found that 20 (13%) of the cases carried a MYD88 mutation. 70% were ABC/non-GCB (nGCB)-DLBCL, 15% germinal center B cell DLBCL (GCB), and 15% unknown. Eleven of the 20 (55%) MYD88 mutant DLBCL cases had a TNFAIP3 loss (Fig. 1b). Taking a closer look at those cases, 73% had a MYD88L265P mutation (ABC/nGCB-DLBCL, n = 5; GCB-DLBCL, n = 1; and unknown, n = 2) followed by 18% with MYD88S243N (ABC/nGCB-DLBCL, n = 1 and GCB-DLBCL, n = 1), and 9% with MYD88M233T (ABC/nGCB-DLBCL, n = 1). TNFAIP3 loss was the most frequent (55%) copy number loss in our cohort of MYD88 mutant cases (Supplemental Figure 2A). Other frequent losses in our MYD88 mutant cases where CDKN2A (50%), ARID1B (45%), ZNF292 (45%), and PRDM1 (45%). Other genetic events that frequently occurred in combination with MYD88 mutations included copy number gains of BCL2 and KLHL14 (45%) and mutations in KMT2D and CD79B (40%) (Supplemental Figure 2B and C). For our analysis of WM, we used genetic data from 29 cases and found that 97% (n = 28) carried a MYD88 mutation. Of those, 28% (n = 8) had a TNFAIP3 loss (Fig. 1c). The high incidence of TNFAIP3 loss combined with MYD88 mutations suggests a relationship between these genetic events. Statistical analyses of our data suggest that TNFAIP3 loss and MYD88 mutations are positively correlated in DLBCL (p = 0.017), but not WM (p = 1.0) WM,
although our sample size was small. We were able to validate our findings in DLBCL using available data from the Chapuy et al. study which reported 6q or 6q23.3 loss and MYD88 mutation in n = 304 patients (p < 0.0001). Together these data suggest that TNFAIP3 loss is one of the most frequent genetic alterations in MYD88 mutant DLBCL and WM.

To further understand the cellular consequence of MYD88L265P in combination with TNFAIP3 loss in human models of WM and DLBCL, we used TALENs genome editing technology to genetically modify the WM cell line MWCL and the ABC-DLBCL cell line HBL-1, both of which harbor a MYD88L265P mutation, but have a wild type TNFAIP3. To introduce a TNFAIP3 loss in those cell lines, we designed a unique pair of TALENs to target exon 5 of the TNFAIP3 gene to induce a double strand break resulting in a base pair deletions in exon 5 (Fig. 1d). TALENs and a GFP co-expressing plasmid were transfected into the cell lines and GFP positive cells were single cell sorted, expanded, and screened for A20 loss by flow cytometry.

Fig. 1 Frequency of MYD88 mutations in combination with TNFAIP3 genetic alterations in DLBCL and WM patients. a Schematic representation of the MYD88 signaling pathway. b Venn diagram of MYD88 mutant and overlapping TNFAIP3 loss in DLBCL patients. DLBCL MYD88 mutation status was assessed by whole exome sequencing (WES) and TNFAIP3 loss was determined by WES (n = 56) and OncoScan (n = 89) data. c Venn diagram of MYD88 mutant and overlapping TNFAIP3 loss in WM patients. MYD88 status in WM patients was done by Sanger sequencing and TNFAIP3 status was detected by gene copy number analysis. d Schematic representation of the genomic structure of the TNFAIP3 gene. Exons are indicated by light blue boxes. TALENs were designed to target sequences in exon 5 and sequences of TALENs binding sites are highlighted in bold red. e Western blot analysis showed a reduction of TNFAIP3 (A20) in the newly generated cell lines, compared to their wild type counterpart. β-Actin was used as a loading control (n = 3). f Western blot analysis of MYD88 and TRAF6 in MWCL and HBL-1 A20 knock out cell lines and their wild type counterpart. β-Actin was used as a loading control (n = 3).
western blot (Fig. 1e). Western blot analysis showed 80% reduction of A20 in the WM cells and a nearly complete loss of A20 in the DLBCL cells, hereafter referred to as MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\), compared to their wild type counterpart. To ensure that A20 knockdown did not impact the expression of key MYD88 signaling molecules, we measured the expression of MYD88 and TRAF6 in each of the cell lines. We did not detect any changes in MYD88 or TRAF6 expression suggesting that the MYD88 signaling pathway is fully intact in MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) cells (Fig. 1f) ensuring that signaling defects are specific for A20 loss.

**Loss of A20 enhances MYD88\(_{L265P}\) -driven signaling and contributes to ibrutinib resistance**

To explore the possibility that loss of A20 activates MYD88\(_{L265P}\)-driven signaling, we measured the impact of TNFAIP3 deletion on activation of p38 and NF-κB in the MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) cell lines. Western blot analysis revealed significant upregulation of phosphorylated p38 (2.02-fold in MWCL-A20\(_{ko}\); \(p = 0.006\) and 1.71-fold in HBL-1-A20\(_{ko}\); \(p = 0.03\)) and NF-κB (1.44-fold in MWCL-A20\(_{ko}\); \(p = 0.05\) and 1.22-fold in HBL-1-A20\(_{ko}\); \(p = 0.03\)) when compared to the wild type cell line controls (Fig. 2a, b). Graphical representation of multiple experiments is shown in the lower panel of each figure. These studies suggest that loss of A20 in human models of WM and DLBCL drives and enhanced MYD88\(_{L265P}\)-driven signaling.

A recent study on DLBCL patients\(^{17}\) suggests that TNFAIP3 inactivation negatively impacts therapeutic responses to ibrutinib, a BTK inhibitor known to inhibit B-cell receptor and NF-κB signaling\(^{26,27}\). Therefore, we next wanted to determine if ibrutinib responses were impacted by the loss of A20 in our cell models. The MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) cell lines, along with their respective controls, were treated in a dose-dependent manner with ibrutinib and proliferation was measured after 48 h. We did not detect any significant differences in the response to ibrutinib in the WM cell lines (Fig. 2c, left panel). However, HBL-1-A20\(_{ko}\) was significantly (\(p < 0.05\)) more resistant to ibrutinib single agent therapy than HBL-1 wild type cell line (Fig. 2d, right panel). This data suggests that DLBCL patients with a MYD88 mutation and an A20 loss are more resistant to ibrutinib single agent therapy.

**Loss of A20 induces upregulation of NF-κB target genes**

To further study the effects of A20 knock out, we performed RNASeq analysis of MWCL-A20\(_{ko}\), HBL-1-A20\(_{ko}\), and their matched control cell lines. The RNASeq data revealed that the NF-κB target genes, IL-6, CXCL10, BCL2, and MYC are upregulated in the A20 knock out cell lines (Fig. 3a). To validate the RNASeq findings, we performed quantitative PCR experiments on the cell lines and confirmed that IL-6 and CXCL10 were significantly upregulated in both MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) compared to cells with intact TNFAIP3 (Fig. 3b). BCL2 and MYC were significantly upregulated in the HBL-1-A20\(_{ko}\) cells and showed a slight upregulation in the MWCL-A20\(_{ko}\). This data indicates that TNFAIP3 loss drives upregulation of NF-κB target genes in DLBCL and WM.

**Upregulation of NF-κB target gene protein expression in A20 knock out cells**

To further validate RNA expression data, we performed western blot analysis of BCL2 and MYC (Fig. 4a) and confirmed that both are significantly upregulated in the HBL-1-A20\(_{ko}\) cell line. Graphical representation of multiple experiments is shown in the lower panel of each figure. There were no changes in the protein levels of BCL2 and MYC in the MWCL-A20\(_{ko}\), suggesting that loss of A20 in DLBCL and WM may have different biologic effect in these two forms of NHL. Another possibility that may explain the differences in BCL2 and MYC protein upregulation in our knock out cell lines is the residual A20 protein expression found in the MWCL-A20\(_{ko}\) compared to the HBL-1 cells. Therefore, we generated a second WM cell line with A20 loss (BCWM-A20\(_{ko}\)) that has a 94% reduction in A20 expression (Supplemental Figure 3A). Again, we did not detect any changes in BCL2 or MYC (\(p = 0.1104\) and \(p = 0.7624\), \(n = 3\)) protein expression in the BCWM-A20\(_{ko}\) compared to the control cells (Supplement Figure 3B and C). This data further supports the idea that A20 loss has differential effects in WM and DLBCL, which may be expected due to disease heterogeneity.

To assess the expression of IL-6 and CXCL10, we carried out Luminex single plex assays (Fig. 4b). Cells were cultured for 48 h in standard media and supernatants were analyzed. Both IL-6 and CXCL10 were significantly upregulated in the media of MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) cell lines compared to their wild type counterpart (Fig. 4b). These data validate our previous findings and indicate that loss of A20 drives expression of BCL2, MYC, IL-6, and CXCL10.

**MYD88\(_{L265P}\) and TNFAIP3 loss drives the JAK/STAT pathway**

MYD88\(_{L265P}\) has been shown to drive autocrine expression of cytokines resulting in the activation of the JAK/STAT pathway\(^{26,28}\). Therefore, we next sought to determine if our newly generated A20 knock out cell lines had increased STAT3 activation, a downstream target of IL-6. Western blot analysis of phosphorylated STAT3 levels showed that MWCL-A20\(_{ko}\) and HBL-1-A20\(_{ko}\) cell lines had significantly higher levels of p-STAT3 (1.47-fold in MWCL-A20\(_{ko}\); \(p = 0.0176\) and 1.46-fold in HBL-1-A20\(_{ko}\); \(p = 0.0114\)) (Fig. 5). Graphical representation of
multiple experiments is shown in the lower panel. These data suggest that upregulation of IL-6 induced by A20 loss drives activation of the JAK/STAT in WM and DLBCL cells.

Discussion

In this study, we sought to better define the genetic profile and biologic impact of MYD88 mutations in combination with TNFAIP3 loss in WM and DLBCL. MYD88L265P drives cell proliferation, but it is rapidly shut down by a TNFAIP3 negative feedback loop, suggesting an important relationship between these proteins. Our data show that a large percentage of DLBCL and WM cases that have a MYD88 mutation also harbor a TNFAIP3 loss, 55% DLBCL and 28%, respectively. These data are supported by previously published work showing that 11–55% of the MYD88 mutant DLBCL have a TNFAIP3 genetic alteration and 35–50% of WM patients harbor a TNFAIP3 loss. The importance of gaining insight of the impact of MYD88 mutations in combination with other genomic events in lymphoma is further supported by recent findings showing a correlation between MYD88 mutation status and poor outcome in DLBCL. Furthermore, in the study performed by Reddy et al. MYD88 mutations alone are a prognostic factor for the ABC-DLBCL subgroup. However,
additional studies counter these data and suggest a need for additional genetic and clinical analysis to further define the impact by MYD88 mutations in combination with other genetic alterations\textsuperscript{30,34–36}. Two recent large-scale studies shed new insight on how genomic alterations contribute to overall survival in DLBCL patients\textsuperscript{25,37}. Both analyses define novel and genomically unique subgroups of patients with inferior overall survival, MCD\textsuperscript{37} and C5\textsuperscript{25}. Both MCD and C5 share genetic enrichment for MYD88 and CD79B mutations. In the MCD classification, 2.9% of
the patients also have a TNFAIP3 loss, where 46% of MYD88 mutant patients in the C5 group either have a 6q or 6q23.3 loss. Between these studies, it is not clear why there is such a large discrepancy in the rate of TNFAIP3 loss in the MYD88 mutant cases, although our study is in line with the 46% described by Chapuy et al. as well as other publications25,30. This variability is most likely due to the use of different copy number analysis platforms as well as tissue samples type (i.e., fresh frozen vs FFPE). Taken together, this data supports the possibility that loss of TNFAIP3 contributes to the MYD88 effect on outcome and future studies should further evaluate the clinical significance.

Using TALEN genome-editing technology, we were able to generate cell lines that allow for functional characterization of causal genetic variants. One benefit to genome editing of cell lines is that it allows for a direct comparison of a specific alteration in the same genetic background. This system also permitted us to design a genetic model in both DLBCL and WM cell lines, allowing for validation of our results across multiple cell lines. Using the MWCL-A20ko and HBL-1-A20ko, we were able to show that loss of TNFAIP3 enhances MYD88L265P-driven NF-κB and p38 signaling resulting in increased expression of NF-κB target genes IL-6 and CXCL10, known NF-κB target genes2,32,38,39, have both been shown to be significantly upregulated in WM and DLBCL, and higher serum levels correlate with an inferior survival38,40–43. Interestingly, in a new T-cell specific A20 knock out model, serum levels of CXCL10 were significantly increased supporting the idea that CXCL10 expression is regulated by A2044. The role of CXCL10 in B cell lymphomas has not been studied well, but it is known that its receptor CXCR3 is expressed on a small subset of B cells45,46. Additionally, we show
that TNFAIP3 loss contributes to ibrutinib resistance in an ABC-DLBCL cell line. This data aligns with other studies showing that genetic aberrations contribute to ibrutinib resistance in hematological malignancies. A recent study by Kuo et al. showed that ibrutinib resistance is marked by BCL2 upregulation and that combining ibrutinib with ABT-199, a BCL2 inhibitor, could overcome resistance in DLBCL. Together, these studies suggest that genetic analysis of tumors may inform therapeutic choices and highlights the potential importance of individualized therapy based on genetic profiles.

IL-6 regulation by A20 has been shown in mouse models where TNFAIP3 was depleted in B cells. Mice lacking A20 had higher mRNA levels and secreted more IL-6 than mice having an intact TNFAIP3 locus after B cell activation. Gene expression analysis of WM patients has shown that IL-6 is one of the most increased expressed genes and A20 is lost in a high percentage of WM patients indicating that A20 controls cytokine production in WM. A previous study identified a subgroup of ABC-DLBCL with a high autocrine IL-6 production driving phosphorylation of STAT3. MYD88 mutations were highly enriched in this subgroup showing that IL-6 production is driven by dysregulated MYD88 signaling. However, our data indicate that this dysregulation can be further driven by TNFAIP3 loss in patients with MYD88 mutations.

The importance of IL-6 and the autocrine mechanism by which IL-6 induces STAT3 activation in DLBCL has been shown in previous studies. STAT3 overexpression is a prognostic marker for overall survival in DLBCL and high STAT3 and phosphorylated STAT3 in the nucleus correlates with inferior survival on those patients. Furthermore, it has been shown that STAT3 upregulation contributes to IgM secretion in WM, which can cause severe complication in WM patients. A recent study demonstrated how STAT3 acts as an activator of several oncogenic pathways as well as a suppressor for apoptosis. Additionally, it has been shown that MYC and BCL2 overexpression significantly correlates with high phosphorylation of STAT3 in DLBCL. Our data support these findings and extend to our mechanistic understanding of how MYD88 and A20 signaling can contribute to IL-6 secretion, STAT3 activation, and expression of pro-survival genes.

BCL2 and MYC are both well-studied oncogenes and are targets of recurrent chromosomal breakpoints in lymphomas. If both MYC and BCL2 undergo rearrangement at the same time, they are referred to as double hit lymphoma, and often fall into the GCB-DLBCL subgroup. However, there is also another subclass which is referred to as double expressers, where MYC and BCL2 are overexpressed independent of genetic rearrangement, and often fall into the ABC-DLBCL subgroup. A recent study showed that BCL2 and MYC expression is significantly associated with MYD88 mutations in the ABC-DLBCL subgroup, however this study only looked at the MYD88 mutation status and no other genetic alterations in combination to BCL2 and MYC status. Our data indicate that TNFAIP3 loss together with MYD88L265P drives upregulation of anti-apoptotic and cell survival signaling in DLBCL. It would be interesting to see if double expressing ABC-DLBCL also harbor a MYD88 mutation or TNFAIP3 loss or both together since our data indicate that both genetic aberrations contribute to BCL2 and MYC expression in ABC-DLBCL. On the other hand, recent studies have shown that expression levels of the BCL2 family in WM patients is almost the same as in healthy controls and that WM cell lines treated with ABT-737, a BCL2 inhibitor, lack sensitivity. Together, this data supports the idea that the biological pathways activated by MYD88 and TNFAIP3 loss in WM and DLBCL are unique. In summary, we have established a new WM and DLBCL cell line model that mimics the effect of the MYD88L265P mutation in combination with a loss of the TNFAIP3 gene and A20 expression. We show that loss of TNFAIP3 results in a higher baseline phosphorylation of NF-κB, p38, and STAT3. Additionally, loss
of TNFAIP3 impacts expression of IL-6 and CXCL10. Overall, results from this study contribute to our understanding of MYD88-driven lymphomas, suggests a possible clinical implication for those individuals that harbor both a MYD88 mutation and a loss of TNFAIP3, and also provides us with a useful model to study novel therapeutic strategies in patients who harbor these genetic variants.

Acknowledgements
This work was supported in part by the National Institutes of Health (PSD CA097274 to J.R.C. and A.J.N.; CA212162 to A.J.N. and J.R.C.), and the Predolin Foundation (to A.J.N.). We would like to thank the Mayo Clinic Center For Individualized Medicine for their support of the Bioinformatics Program and Biomarker Discovery Program. We would also like to thank the Mayo Clinic Center for Cell Signaling in Gastroenterology for helping to design the TALEN construct.

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KW, J.R.C., and A.J.N designed, analyzed and interpreted the data, and drafted the paper. K.W., M.M., S.V., Y., P.T.G., H.R.S., and M.J.M. performed experiments, analyzed data, and edited the manuscript. E.B., S.L.S., T.E.W., and S.M.A. contributed to the paper. K.W., M.M., S.V., Y.A., P.T.G., H.R.S., and M.J.M. performed experiments, analyzed data, and edited the manuscript. E.B., S.L.S., T.E.W., and S.M.A. contributed to the paper. K.W., J.R.C., and A.J.N. designed, analyzed and interpreted the data, and drafted the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information
accompanies this paper at (https://doi.org/10.1038/s41408-018-0130-3).

Received: 2 May 2018 Revised: 4 August 2018 Accepted: 9 August 2018

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