Targeting Myddosome Assembly in Waldenstrom Macroglobulinaemia

MYD88 mutations are expressed in Waldenstrom Macroglobulinaemia (WM) (95–97%), immune-privileged lymphomas (50–80%), activated B-cell (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) (15–30%), marginal zone lymphoma (6–10%) and chronic lymphocytic leukaemia (CLL) (3–8%) (Montesinos-Rongeri *et al*, 2011; Ngo *et al*, 2011; Pasqualucci *et al*, 2011; Puente *et al*, 2011; Treon *et al*, 2012; Martínez *et al*, 2014). In WM, nearly all cases express the $MYD88^{L265P}$ mutation (Treon *et al*, 2012); 50–80% of IgM MGUS patients express $MYD88^{L265P}$ by allele-specific polymerase chain reaction, and its expression is associated with an increased risk of malignant progression (Varettoni *et al*, 2013; Xu *et al*, 2013).

The importance of mutated *MYD88* in supporting tumour cell survival in ABC DLBCL and WM was established by *MYD88* gene knock-down and/or over-expression studies (Ngo *et al*, 2011; Treon *et al*, 2012; Yang *et al*, 2013). MYD88 dimerization is necessary for assembly of the Myddosome, a structure composed of MYD88 dimers that recruit and activate interleukin-1 receptor-associated kinase (IRAK) 4, which in turn recruits and activates IRAK1 or 2. The MYD88:IRAK4:IRAK1/2 complex can then trigger canonical nuclear factor-*kappa* B (NFκB) growth and survival signalling (Ngo *et al*, 2011; Yang *et al*, 2013). The Myddosome can also activate Bruton Tyrosine Kinase (BTK), which can promote canonical NFκB signalling in mutated *MYD88* WM cells (Yang *et al*, 2013).

The MYD88 protein is composed of an N-terminal death domain (DD) spanning amino acids 40-119, an intermediate linker domain (ID) that spans amino acids 120–173, and a C-terminal Toll/IL1 receptor (TIR) domain that encompasses amino acids 174–309 (NCBI NP_002459.2). Upon ligand binding to TLR/IL1R, the TIR domain facilitates binding to the receptor complex, and promotes MYD88 dimerization. Loiarro *et al* (2013) used site-directed mutagenesis of conserved residues within the *MYD88* TIR domain to analyse their impact on MYD88 protein signalling. Their work revealed that mutation of Glu^{196}, Ser^{257} and Arg^{301} [per National Center for Biotechnology Information (NCBI)NP_002459.2 annotation] interfered with TIR-mediated MYD88 dimerization, IRAK recruitment, and reduced NFκB activation. The over-expression of two MyD88 ($MYD88^{164–202}$ and $MYD88^{181–202}$) mini-peptides that included Glu^{196} in wild-type *MYD88* ($MYD88^{WT}$) THP-1 human monocyte cells also blocked TLR/IL1R triggered MYD88 dimerization and NFκB activation.

There is evidence that the MYD88 DD domain can also facilitate MYD88 dimerization and MYD88-IRAK4 heterodimerization. Structural modelling has highlighted Ser^{47} and Arg^{111} (per NCBI NP_002459.2 annotation) as contributors to Myddosome assembly, IRAK4 recruitment and NFκB signalling, though without an intact TIR domain such interactions appear weak (George *et al*, 2011). The understanding of how MYD88 and IRAK4 DD domains interact to support Myddosome assembly has been aided by crystal structure modelling of the MYD88:IRAK4:IRAK2 DD complex that shows Val^{56}, Ala^{57}, Glu^{65}, Tyr^{71}, Ile^{74} and Arg^{75} (per NCBI NP_002459.2 annotation) of MYD88 as critical determinants of MYD88-IRAK4 binding (Lin *et al*, 2010).

In contrast to native MYD88, mutated MYD88 protein can assemble without external stimuli, and trigger constitutive NFκB activation (Ngo *et al*, 2011; Treon *et al*, 2012; Yang *et al*, 2013). All activating *MYD88* mutations observed in B-cell malignancies, including $MYD88^{L265P}$, reside in the TIR domain. Relative to $MYD88^{WT}$, mutated MYD88 protein shows enhanced IRAK4 and IRAK1 binding, and NFκB activation. While the precise structural interaction(s) that promote constitutive MYD88 homodimeric and IRAK heterodimeric interactions remain to be clarified in *MYD88* mutated cells, the potential to block Myddosome self-assembly may be clinically relevant. We therefore sought to disrupt Myddosome signalling by systematic evaluation of mini-peptides designed to compete with MYD88 TIR and DD domain interactions, and studied their impact on downstream signalling and survival in *MYD88* mutated WM cells.

Oligonucleotides corresponding to the TIR and DD domain peptide sequences (Fig 1A) were cloned into Lenti-X™ Tet-One™ (Clontech Laboratories, Inc., Mountain View, CA, USA) inducible vector with an N-terminal GFP fusion sequence: MYD88 TIR domain: $P_{181-202}$ (sense: CTATTG CCCCAGCGACATCCAGTTT, anti-sense: CAGTCGATAGTT TGTCTGTTCCAGTTGC), $P_{256-292}$ (sense: CTCTCTCCAG GTGCCCATCAGAA, anti-sense: AGGGTTGGTGTAGTCG-CAGACAGT), $P_{190-197}$ (oligonucleotide: GAGATGATCCGGC AACTGGAACAG), $P_{191-198}$ (oligonucleotide: ATGATCCGGC AACTGGAACAGACA), $P_{195-202}$ (oligonucleotide: CTGGAA-CAGACAAACTATCGACTG), $P_{295-302}$ (oligonucleotide: AAA TCTTGGTTCTGGACTCGCCTT), and MYD88 DD domain: P_{40-85} (sense: CGGCGCCGCCTGTCTCTGTT, anti-sense: GC CAGTGGGGTCCGCTTGT). Molecular modelling depicting the surface areas and ribbon structures of the MYD88 TIR

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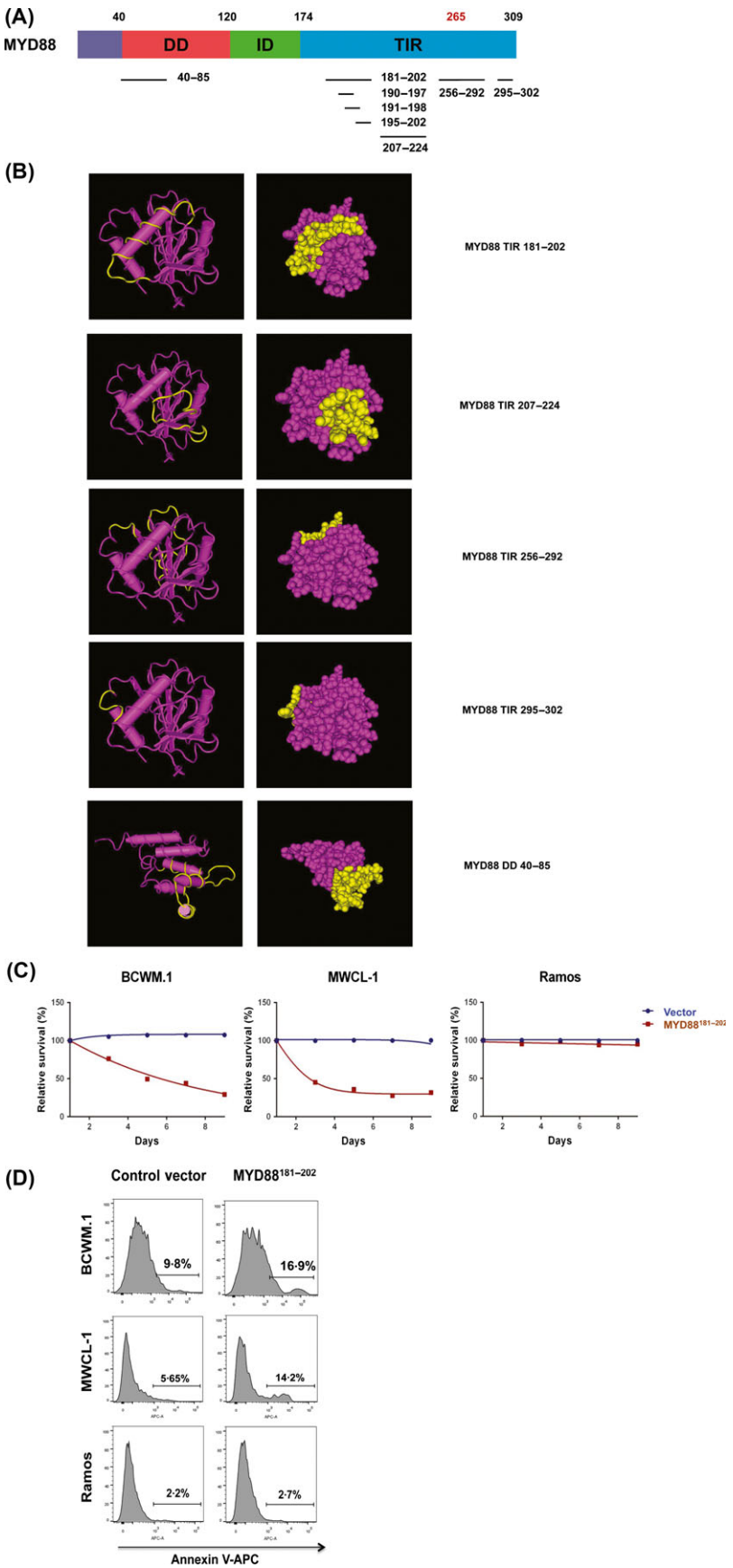

Fig 1. MYD88 TIR domain dimerization supports pro-survival signalling in *MYD88* mutated cells. (A) Structure of MYD88 protein identifying death domain (DD), intermediate domain (ID) and Toll/IL-1 receptor (TIR) domain, and regions targeted for interference by mini-peptide constructs. (B) Molecular modelling depicting surface areas and ribbon structures of the MYD88 protein TIR and DD domain regions that were targeted for interference by mini-peptide constructs based on NCBI MMDB 108985 (TIR) MMDB:82515 (DD) crystal structure models. (C) Results of AlamarBlue® growth assays following transduction of *MYD88* mutated BCWM.1 and MWCL-1 or *MYD88* wild-type Ramos cells with control vector or vector coding for $MYD88^{181-202}$ mini-peptides. (D) Apoptotic changes following 72 h of doxycycline induction by Annexin V staining on GFP^{+} BCWM.1, MWCL-1 and Ramos cells transduced with control vector or vector coding for $MYD88^{181-202}$ mini-peptides. Representative results from two independent experiments are shown.

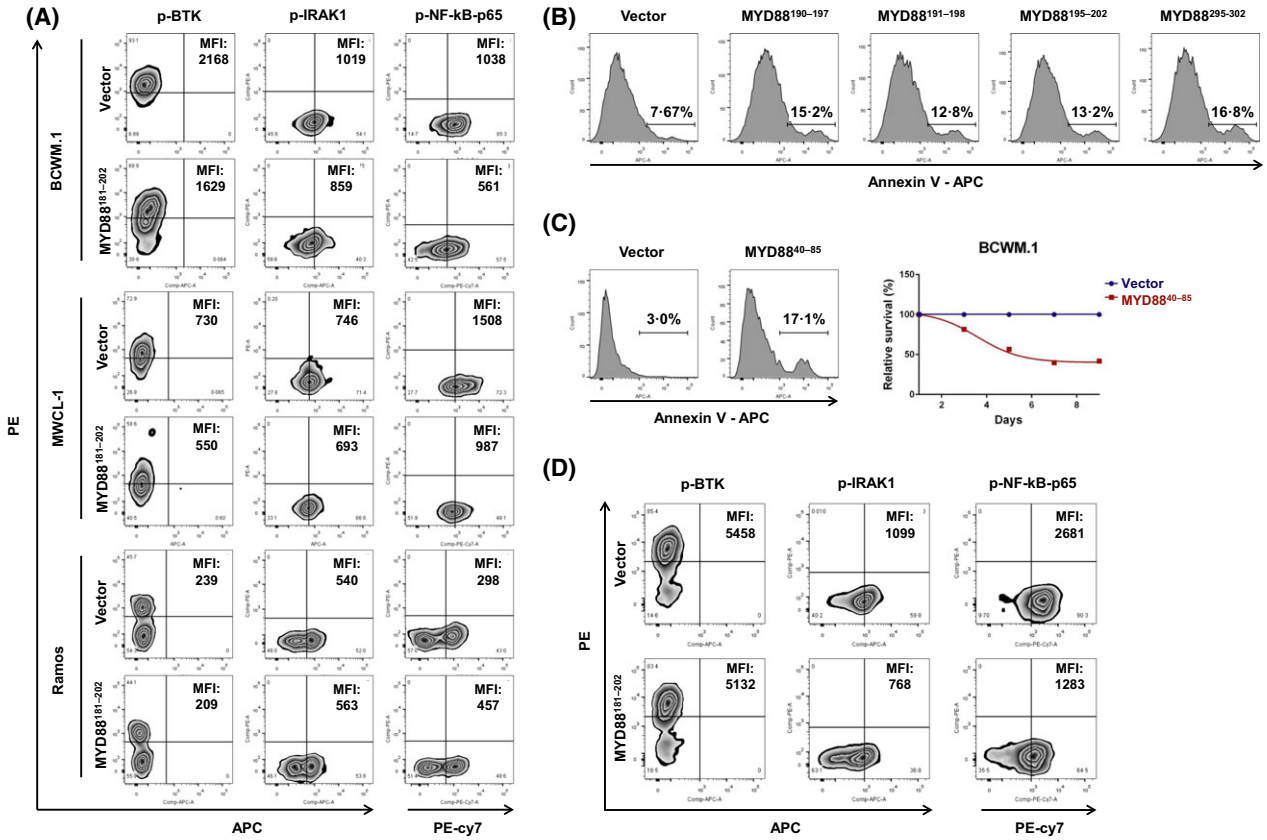

Fig 2. Impact of TIR and DD Domain Mini-Peptides on Pro-Survival Signalling in *MYD88* mutated WM cells. (A) PhosFlow studies to assess changes in pBTK^{Y223}, pIRAK1^{T209} and pNFκB-p65^{S529} activation following 24 h of doxycycline induction for GFP^{+} BCWM.1, MWCL-1 and Ramos cells following transduction with a control vector or vector coding for MYD88$^{181-202}$ mini-peptide. (B) Apoptotic changes following 72 h of doxycycline induction by Annexin V staining on GFP^{+} BCWM.1 cells transduced with control vector or vector coding for 8-mer MYD88 mini-peptides that target Glu^{196} and Arg^{301}. (C) Results of apoptosis assay following 72 h of doxycycline induction, and AlamarBlue® growth assays following transduction of BCWM.1 cells with control vector or vector coding for a mini-peptide that targets the DD domain (MYD88$^{40-85}$). (D) PhosFlow studies to assess changes in pBTK^{Y223}, pIRAK1^{T209} and pNF-κB-p65^{S529} activation following 24 h for GFP^{+} BCWM.1 cells following transduction with a control vector or vector coding for a DD domain (MYD88$^{40-85}$) mini-peptide. Representative results from two independent experiments are shown.

and DD domain regions targeted for interference by mini-peptide constructs based on NCBI MMDB 108985 (TIR) MMDB:82515 (DD) crystal structure models are shown in Fig 1B. Following lentiviral infection and puromycin selection in tetracycline-free media, *MYD88*^{L265P} mutated BCWM.1 and MWCL-1 or *MYD88*^{WT} Ramos cells were treated with doxycycline. Cell survival was then assessed on sorted GFP^{+} cells with AlamarBlue® (Invitrogen, Carlsbad, CA, USA). Annexin V staining (BD Biosciences, San Jose, CA, USA) was used for detecting apoptotic changes on GFP^{+} cells by flow cytometry. To detect changes in MYD88 downstream signalling, PhosFlow® analysis was performed for pBTK^{Y223}, pNFκB-p65^{S529} (BD Biosciences) and pIRAK1^{T209} (Abcam, Cambridge, MA, USA) on GFP^{+} cells (Yang *et al*, 2013).

Given that mini-peptides expressing Glu^{196} within the TIR domain blocked TLR/IL1R-triggered MYD88 dimerization and NFκB activation in *MYD88*^{WT} THP-1 human monocytes, we first examined a mini-peptide (MYD88$^{181-202}$) that targeted this region in *MYD88*^{L265P} mutated BCWM.1 and MWCL-1 cells, and *MYD88*^{WT} Ramos cells (Figs 1A,B).

Expression of MYD88$^{181-202}$ blocked growth of BCWM.1 and MWCL-1 cells, but did not impact growth of *MYD88*^{WT} Ramos cells (Fig 1C). Annexin V staining showed apoptotic changes in *MYD88*^{L265P} but not *MYD88*^{WT} cells transduced with MYD88$^{181-202}$ (Fig 1D). Moreover, pro-survival signalling (pBTK^{Y223}, pIRAK1^{T209}, pNFκB-p65^{S529}) was abrogated in *MYD88*^{L265P} but not *MYD88*^{WT} cells transduced with MYD88$^{181-202}$ (Fig 2A). Expression of smaller mini-peptides (8-mers) that overlapped Glu^{196} (MYD88$^{190-197}$, MYD88$^{191-198}$ and MYD88$^{195-202}$ also showed increased apoptosis at 72 h in MYD88^{L265P} mutated BCWM.1 cells (Fig 2B). In contrast, no sustained growth inhibition of BCWM.1 cells with any of the 8-mer peptides was observed (data not shown). In addition to Glu^{196}, Loiarro *et al* (2013) also showed that mutation of Ser^{257} and Arg^{301} in MYD88

also interfered with TIR-mediated dimerization. To clarify their contribution to MYD88-directed survival signalling, BCWM.1 cells were also transduced with vectors coding for mini-peptides spanning amino acids 256–292 and 295–302. Apoptotic changes relative to control vector were observed in only $MYD88^{295\text{-}302}$ transduced BCWM.1 cells (Fig 2B). Neither $MYD88^{256\text{-}292}$ nor $MYD88^{295\text{-}302}$ impacted $pBTK^{Y223}$, $pIRAK1^{T209}$ or BCWM.1 cell survival, while moderate reductions in $pNF\kappa B\text{-}p65^{S529}$ were observed with only $MYD88^{256\text{-}292}$ (data not shown). Transduction of a mini-peptide interfering with BB-loop interactions ($MYD88^{207\text{-}224}$) also showed weak or no reduction in pro-survival signalling ($pBTK^{Y223}$, $pIRAK1^{T209}$, $pNF\kappa B\text{-}p65^{S529}$), nor were any meaningful apoptotic or sustained growth inhibitory effects observed relative to control vector transduced BCWM.1 cells (data not shown). The findings with $MYD88^{256\text{-}292}$ and $MYD88^{207\text{-}224}$ were particularly instructive, given that most of the described activating *MYD88* mutations are located in either the BB loop, or at position 265.

Further to these experiments, we also sought to clarify the contributions of the DD domain to pro-survival signalling in $MYD88^{L265P}$ mutated WM cells. We therefore transduced BCWM.1 cells with either a control vector or vector coding for a mini-peptide that spanned amino acids 40–85 ($MYD88^{40\text{-}85}$) within the DD domain of MYD88. Expression of the $MYD88^{40\text{-}85}$ mini-peptide in BCWM.1 cells was associated with increased apoptosis, and contributed to sustained growth inhibition (Fig 2C), as well as reduced $pIRAK1^{T209}$ and $pNF\kappa B\text{-}p65^{S529}$ activation (Fig 2D).

The findings therefore suggest that optimal survival signalling of *MYD88* mutated WM cells involves TIR domain residues overlapping Glu^{196}, and possibly residues extending from amino acids 40–85 in the DD domain. Reductions in IRAK and/or BTK signalling probably contributed to decreased WM cell survival, though greater reductions in $pNF\kappa B\text{-}p65^{S529}$ were observed in comparison to pIRAK or pBTK following transduction of $MYD88^{181\text{-}202}$ or $MYD88^{40\text{-}85}$. These findings may indicate either a critical scaffold function for the IRAK and/or BTK proteins beyond their well-established pro-survival kinase activity, and/or the presence of other binding partners that support growth signalling in *MYD88* mutated WM cells. A scaffold function for TLR/IL1R triggered IRAK1 activity has been previously described in $MYD88^{WT}$ cells (Böl *et al*, 2005). A potential scaffold function for the IRAK proteins may also explain why knockdown of IRAK1 or IRAK4 produces higher levels of apoptosis than observed with inhibitors targeting these kinases in *MYD88* mutated WM cells (Yang *et al*, 2013, 2015) Lastly, the findings also provide regions of interest that are conducive to the development of small molecules, peptidomimetics or stapled peptides for potential therapeutic application. Larger peptide sequences (>8-mers) may be needed within the TIR $MYD88^{181\text{-}202}$ domain to achieve sustained growth suppression as observed in our studies. The development of stapled peptides may be particularly suitable, as longer (20-mer peptides) have been developed for therapeutic application by this approach (Walensky & Bird, 2014). In addition, the $MYD88^{181\text{-}202}$ domain has an alpha helical configuration that is ideal for manufacturing stapled peptides (Fig 1B). It is also possible that multiple stapled peptides targeting both the TIR and DD domains could be used to more optimally block Myddosome assembly. In summary, we show that interference of Myddosome assembly within select regions of the TIR and DD domains can impact growth and survival signalling of $MYD88^{L265P}$ mutated WM cells. Our findings provide a framework for developing agents that interfere with Myddosome assembly in WM, and possibly for other lymphoproliferative disorders driven by MYD88-activating mutations.

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Author contributions

XL, GY, and SPT designed the study. GY and XL designed the primers. XL, JGC performed the lentiviral transduction experiments. JC, NT, and GY performed the signalling studies. CJP and JJC provided samples. ZRH, SB, NG, and ST performed data analysis.

Conflict of interest disclosures

The co-authors have no relevant disclosures for this work.

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