nature portfolio

Peer Review File

PIM2 inhibition promotes MCL1 dependency in plasma cells involving integrated stress response-driven NOXA expression

Corresponding Author: Professor Thierry Fest

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Reviewer comments to the manuscript „Uncovering the Role of PIM2 in Modulating MCL1 Dependency and Activation of ISR-Mediated NOXA Expression“ by Marion Haas et al.

The manuscript presents substantial data regarding PIM2 inhibition/knock-down/out and its effect on Bcl-2 family regulated mitochondrial apoptosis signaling pathway. The authors claim that PIM2 inactivation alleviates BAD phosphorylation and activates integrated stress response thereby inducing expression of the pro-apoptotic BH3-only protein NOXA that inhibits MCL-1 resulting in increased (sensitivity to) apoptosis. The authors present a variety of convincing data covering a broad range of biological/biochemical aspects addressed by suitable basic (immunochemical, flow cytometric) and sophisticated methods. Finally, the authors present data to prove the ISR dependent pro-apoptotic (anti-tumoral) activity of combined PIM-inhibition and MCL-1 inhibition by co-applying the ISR-inhibitor ISRIB in a mouse model of femur injected myeloma cells. The results from mouse experiments are well in line (and even succeed) expectations from wet lab experiments.
Despite the overall very positive first impression this reviewer has a lengthy list of concerns, partially regarding essential aspects and data in the manuscript. These concerns are far beyond simple errors and must be addressed before considering publication in a highly renowned journal.

Major Points:
A) The title is misleading. Authors show the effect of PIM inhibition or knock-down – the results might originate from indirect and/or downstream effects.
B) The principle finding that ISR induces NOXA expression has already been published (Cell Death Discovery (2022) 8:215).
C) Co-IP experiments look impressively clean (no detection of heavy or light chain) and efficient. Lysates from knock-out or over-expressing cells would serve as additional control. This is absolutely important when analyzing interaction and activity of Bcl-2 family proteins since authors use NP-40/deoxycholate as detergents (line 763). Conformational change of BAX and interaction with BCL-xL, Bcl-2 has been shown to be induced by non-ionic detergents (J Biol Chem (1997) 272:13829-34). This might also apply for the interaction of other anti-apoptotic and BH3-only proteins.
D) The author´s present a huge set of data. However, there is almost no re-production of published characteristics making it hard to judge whether the investigated aspects are solely responsible for the presented results. Specifically: what happens to cells incubated with PIMi (AZD1208) regarding mTOR (e.g. Blood (2013) 122: 1610–20.), autophagy, cell cycle (e.g. Cancer Res (2008) 68: 5076–85)? At least some of these aspects need to be included to estimate the importance of pBAD, ISR, NOXA in relation.
E) Concerning the differentiation model of B-cells in Figure 1: the authors have any suggestion why NOXA is only significantly upregulated in D4 aBCs and not at later stages of differentiation? Are dead cells increasing during differentiation that are harvested?
F) The inhibitor AZD1208 has been published as pan-PIM inhibitor. Concentrations of AZD1208 in a number of experiments is rather high (µM) – the authors need to exclude off-target effects more rigorously, e.g. by applying the inhibitor to PIM2-knock-out cells.
G) The rational for selecting the indicated cell lines is unclear. Some of the cell lines are not standard and information on mutational status of important proteins is lacking. Since NOXA has been originally published as p53 target, at least p53 mutational status of the investigated cell lines must be shown – especially since the authors conclude that p53 expression is not affected by PIMi (which indicates mutation of p53)!

H) Is it correct, that the percentage of Casp-3 positive cells in Figure 2F/3G is 1% - 3 %? Or is this shown relative to the control? In the methods part the authors state that for Caspase-3 active and TMRM staining (flow cytometry) dead cells were excluded from the detection via DAPI staining (Lane 742). In the same regard, in lane 123 and 124, the authors talking about "full caspase 3 activation". This might be misleading if caspase-3 activation was assessed in viable cells. The authors should clarify how to interpret these data. Does that mean that the data in Figure 2F/3G show that less then 5 % viable cells are positive for caspase-3? Why were dead cells excluded in the first place for assessment of apoptosis after PIMi? Same is true for the TMRM staining, which would then only reflect the shift in membrane potential for viable and not dead cells.
I) Figure 4C/D/E/I: PIMi seems to downregulate MCL-1 or increase its degradation (at least in RPMI8226/U266 cells). However, this is not considered in the schematic model in 4I. Assumingly, the reduced levels of MCL-1 also contribute to the increased apoptosis response? Also, was the reduced level of MCL-1 considered for the subsequent co-IPs showing that MCL-1 is binda more BIM/NOXA (Figure 4G). If not, it remains unclear whether the increased MCL-1 binding results from reduced MCL-1 abundance in these cells. At the very least, the authors should include a discussion of these critical points.
J) Western blots are in part inconsistent regarding expression of central players in apoptosis regulation, most importantly downstream effectors BAX and BAK. In this regard critical point regards to comparably low expression of BAX and BAK in RPMI8226 – how does this fit to apoptosis induction via mitochondrial pathway? Authors should show release of cytochrome c in addition to TMRM. In this regard: what do the authors want to indicate with "right shift of TMRM signal = mitochondrial depolarization" (Fig5AD, supplFIG5EG – missing in supplFIG5F). This reviewer expects a left-shift due to depolarization (loss of deltaPSIm, reduced fluorescence).
K) What is the rational to switch to another cell line (XG7)?
L) Subcellular localization of key proteins (e.g. BAX, MCL-1, cyt c) should be investigated, e.g. by immunofluorescence microscopy.
M) Fig2B: how does marginal induction of NOXA expression fit to highly successful mouse model results? Does apoptosis of U266 cells really rely on NOXA induction? Fig2E: 20 µM – is the inhibitor still specific at this concentration?
N) CRISPR/Cas9-screen is not explained in the methods part – please correct
O) Staining procedure for FACS analysis (Fig6E, 7D) is not included – please correct
P) Fig4B: concentration of BH3-peptide is missing – please correct

Minor points
A) Line62-63: rephrase
B) Line 65: explain CRISPR/Cas9 data
C) Lines 66, 68: rephrase precisely "levels". Take care about gene and protein names.
D) Fig1E and following: order of cell lines differs from supplFIG1C and chages in following figures – this is somewhat confusing. NOXA expression is missing for U266 – please comment.
E) Fig1F: it would be awesome to include relative protein expression level in the table.
F) Line 100: what is "pBAD silencing" supposed to mean. Please rephrase.
G) Line 0:what is "notable expression pattern"? – explain.
H) Fig3, micrco abbreviated with "u" instead of Greek letter.
I) Fig3G: indicate statistical test.
J) Fig4 labelling: RPM8266 vs previously RPMI8226
K) Fig4I: the authors do not show data on subcellular localization. Schematic is not supported by data. Apoptotic threshold has been published decades ago.
L) Fig5B: MCL-1 inhibitor increases expression level of MCL-1 in U266.
M) Fig5D: this reviewer is well aware that WB analyses are not quantitative measurements. However, band intensities for BAX and BAK in Fig5D differ significantly from Fig1.
N) Fig5DF – "right shift" – see above
O) Fig5G: concentration of MCL-1inhibitor missing
P) Figure 6/7: authors should state in the legend what kind of image is shown in the insert (brightfield?, only written once into the image in Figure 7E). Scale bars are missing
Q) Fig7: switching to "two inhibitors" is unfortunate and inconsistent. Please use consistent labeling
R) supplFig3C: explain statistics. Statistical significance seems unlikely.
S) supplFig5E(F)G: see above "right shift".
T) supplFig7F: "Enrichssement" score – please correct. Text in turquoise and light green is almost invisible.

Reviewer #2

(Remarks to the Author)
The study of Haas and colleagues examines the functions of PIM2 in mediating the survival of normal and malignant human plasma cells. They use inhibitors and genetic knockdown approaches to define Pim2 interactions with the mitochondrial survival proteins and the integrated stress response, key drug targets in multiple myeloma. They show strong synergy between PIM and MCL1 inhibitors both biochemical assays and in vivo xenograft models for multiple myeloma.

Overall, this is a very thorough and robust study with many interesting aspects. The data is generally clear, and the authors tackle the problem with several orthogonal approaches that all lead to similar conclusions. I feel this excellent manuscript would be suitable for Nature Communications after some relatively minor changes.

Specific comments.
1. Figure 1. This data is used to set the stage for the study by defining the expression of key pro/anti-apoptotic genes in normal and malignant human plasma cells. For completeness it would be useful to also include data for BFL1/A1 and BMF

(at least at the RNA level) as these genes may also be relevant players for the models proposed.
2. MAJOR POINT. Unless I have missed it, the only data that directly shows the impact of combined PIMi and NOXA loss is Figure 2F, which shows a modest impact for primary ASCs. What is the survival impact for combined PIMi and NOXA knockdown in the MMCL?
3. A related point is the role for BIM in promoting apoptosis. BIM's function gets less attention as it is not transcriptionally induced like NOXA, and has lower affinity for MCL1,but it is still likely a key component of the cell death after PIM/MCL1 inhibition. Parallel PIM/BIM knockdown would answer this question.
4. How is NOXA induced? The data in Figure 3H bottom shows that the block after ISRIB/PIMi is at the transcriptional level, and other data suggest that p53 is not involved. Do the authors at least have a model to explain this?
5. Figure 5F. I am surprised that the value comparing PIMi/MCL1i +/- ISRIB are statistically significant, given the low numbers of experiments and the high variation. Please check these calculations.

Minor comments
6. It appears that the western blots in Figure 1A-B and again in panel E, duplicate the b-actin loading control. This is OK but should be mentioned in the legend.
7. Line 243. Should be suppl Fig 4C.
8. Line257. The conclusion that ISRIB suppresses NOXA recruited to MCL1, appears an overinterpretation as ISRIB blocks Noxa transcription in the first place.
9. The statistical significance p values (*, ** etc) are not defined anywhere in the manuscript.

Reviewer #3

(Remarks to the Author)
What are the noteworthy results?

The manuscript entitled "Uncovering the Role of PIM2 in Modulating MCL1 Dependency and Activation of ISR-Mediated NOXA Expression" details an extensive body of work undertaken by the authors, in normal and malignant plasma cells, that examines the intricacies of the integrated stress response (ISR) axis, and in particular the pivotal role of PIM2 kinase and its interaction with the BCL2 family of proteins, highlighting crucial mechanisms that regulate cell survival and tumor progression.

The manuscript delivers several noteworthy results:
1. Inhibition of PIM2 kinase suppresses pBAD levels and activates the Integrated Stress Response (ISR) pathway inducing NOXA and Caspase 3 in both antibody-secreting cells (ASCs) and multiple myeloma (MM) cell lines.

2. NOXA's role in Caspase 3 activation remains significant regardless of p53 expression and is distinct from the decline in pBAD levels, directly attributed to PIM kinase inhibition.

3. ISRIB inhibition of the ISR in combination with AZD1208 or antisense morpholino PIM inhibition in ASCs, suppresses ATF4/CHOP expression, prevents NOXA induction and Caspase 3 activation independently of pBAD inhibition (Figure 3G) and improves cell viability (Figure 3H and Supplemental Figure 3C).

4. PIM inhibition triggers a cascade of events (Figure 4I) which results in the loading of pro-apoptotic BH3-only proteins onto MCL1. Increased BAD binding to BCL-XL frees BIM and BAK, relocating them to the mitochondrial membrane where they engage with MCL1 along with NOXA. MCL1 thus becomes saturated with proapoptotic BH3-only proteins which accentuates apoptotic priming.

5. PIM inhibition facilitates more effective mitochondrial depolarization induced by MCL1 inhibitor AZD5991, particularly in AZD5991-sensitive MM cell lines. Specifically, PIM2 depletion, but not PIM1 via morpholinos amplifies the response to AZD5991-induced mitochondrial depolarization. PIM inhibition is required to free BAK from MCL1 and prevents BAK and BIM binding to BCL-XL thus activating BAX, triggering MOMP, mitochondrial depolarization, and cytochrome c release, even at lower doses of MCL1 inhibitor in susceptible MM cell lines. PIM inhibition alleviates resistance to MCL1 inhibition.

6. In vivo, enhanced myeloma xenograft regression via the synergistic effect of combining PIM and MCL1 inhibitors, demonstrates improved outcomes compared to single-agent treatments. These data provide a rational for co-targeting treatment strategies in MM.

Will the work be of significance to the field and related fields?
How does it compare to the established literature?

Yes this large body of work, that examines in-depth the role of PIM2 inhibition in apoptotic priming, adds significant value to the field of PIM kinases and targeted therapies for the treatment of multiple myeloma (MM) and other cancer types. It was previously highlighted in the literature that PIM2 is highly expressed in MM, mediating proliferation and promoting cell survival. It also has a key role in the bone destruction typically seen in MM and therefore constitutes a promising therapeutic target. However, PIM2's function in MM is multifaceted and a thorough understanding of all its roles is required to enhance drug development and guide optimal treatment strategies that can effectively cause cell death without activation of alternate resistance pathways. It will be important to elucidate any conferred functional overlap with PIM1 and PIM3 which has not previously been investigated in detail.

The established literature does highlight all 3 PIM kinases are expressed in MM cells and the PIMs act as pro-survival factors to phosphorylate Bcl-2-associated agonist of cell death (BAD) and pre-vent apoptosis. PIM kinases are upregulated in the CD138 + fraction of patient bone marrow. High PIM2/3 protein expression, across the majority of a panel of MM cell lines, was demonstrated by Ramachandran, J., et al. PMID: 27564460 (Figure 1b). Single transient knockdowns of PIM1/2/3 in MM1.S cells via siRNA transfection functionally separated the three isoforms. PIM2 knockdown alone had significant effects on cell survival at 24 and 48 h after transfection and was the only isoform implicated in the activation of the apoptotic pathway (Figure 2) whereas PIM 1 and 3 did not result in apoptosis.

The authors state that "…PIM2 inhibition activates the ISR-related ATF4/CHOP axis, leading to NOXA protein expression concurrent with pBAD inhibition. This represents the first evidence of PIM2's involvement in the integrated stress response (ISR) pathway, expanding our understanding of its pivotal role in regulating cell survival in PCs". However, previously in prostate cell lines, the Kraft group demonstrated that PIM inhibition transcriptionally increased levels of the BH3 protein Noxa by activating the unfolded protein response (UPR), lead to eIF-2α phosphorylation and increased expression of CHOP. Increased levels of Noxa also inactivated the remaining levels of Mcl-1 protein activity PMID: 22080570. This previous data should be highlighted here in this manuscript by the authors.

Does the work support the conclusions and claims, or is additional evidence needed?

For the most part the work supports the conclusions and claims however authors should address the following:
The pan-PIM inhibitor AZD1208 is used throughout and authors state in line 56 "…in parallel with a PIM inhibitor targeting PIM2…" and line 105 "To explore this relationship, we examined the impact of PIM2 inhibition using the PIM inhibitor AZD1208" – all 3 PIM kinases will be inhibited by AZD1208 so PIM2 alone not inhibited by AZD1208.
The SSO experiments target both PIM1 and PIM2 but not PIM3 which has been shown previously to be highly expressed across the MM cell lines used. Although it has been shown previously that PIM2 might by the only PIM kinase regulating apoptosis the authors should address the fact that all 3 PIM kinases are expressed in MM cells and that there is evidence that there is compensatory potential between PIM kinases. The data would benefit from the addition of PIM1 and PIM3 protein expression data in the panel of MM cell lines (Figure 1E) or at least to highlight previous published data in PMID: 27564460 (Figure 1b). Also, authors should comment on the OPM2 cell line which has v weak PIM2 expression (low MCL1 & low NOXA) and very high pBAD levels, compared to the other MM cell lines (Fig 1E/F). Interestingly, PMID 27564460 (Figure 1b) shows OPM2 cells have low PIM1/PIM2 expression, high PIM3 expres-sion and low pBAD expression compared to those with higher levels of PIM2.
Authors show pBAD is decreased with PIMi in OPM2 cells (Figure 2b) and the IC50 dose for AZD1208 is 11.2uM while U266 cells have high PIM2 and MCL1 protein expression & IC50 dose for AZD1208 is 12.2uM. As mentioned above PMID: 27564460 (Figure 1b) shows OPM2 cells express high PIM3 expression; the authors should comment on whether PIM3 might also activate BAD in this cell line. It would be interesting to examine the effect of SSO PIM1/PIM3 & SSO PIM2 in OPM2 cells and determine whether the response to PIMi, siNOXA are similar to that seen in U266 and RPM8226 cells.
Authors should clarify differences in PIM2 isoform expression in RPMI8226 cells in Figure 1e (all 3 expressed) and Figure 2c (CTL1 and CTL2 mainly 34kD expressed)?
Can authors comment on Figure 2A which has low levels of PIM1 expression with SSO PIM1 and complete inhibition of PIM2 with SSO PIM2? Interestingly, NOXA expression does increase slightly with SSO PIM1 (Figure 2A, 3B & 3H) albeit that SSO PIM2 has a larger increase in NOXA expression. Similarly Figure 2C cell Line RPM8226 with SSO PIM1 has a slight increase in NOXA & cleaved caspase expression although pBAD does not decrease.

Are there any flaws in the data analysis, interpretation and conclusions?
I have not identified any significant flaws in the data analysis, interpretation or conclusions in this manuscript

Do these prohibit publication or require revision?
Minor revisions as detailed above and below.

Is the methodology sound? Does the work meet the expected standards in your field?
Yes the methodology is sound and the work is of an exceptional high standard in the field.

Is there enough detail provided in the methods for the work to be reproduced?
Yes there is enough detail provided in the methods for them to be reproduced.

Further edits and Typos:
Figure 2c & 2e title of graph should be "RPMI8226" not "RPM8226"
Figure 3a "Noxa" not "lOXA" and include PIMi (24hrs) on graph on right
Figure 4I "Hight" to "High"
Figure 5C &5D Image for BAK in RPMI cells &XG7 cells has been cropped too close to 23kD band removing part of the upper band?
Figure 3E is there a pBAD image for U266 cell line?
Figure 3F is the casp3 image for RPMI8226 cell line?

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This reviewer appreciates the work and effort the authors have put into improving their manuscript and responding to this reviewer´s concerns. The authors have satisfactorily replied to most of this reviewer´s concerns. However, some replies, naturally, are dissatisfying.
Nevertheless, this reviewer feels that the improvements and replies now justify the publication of the manuscript.

Some minor comments are listed below:
a) Labelling in figures throughout seems to be partially imported as pixel-based image. This is unfortunate since sub-figures are rather small. When zooming in, text becomes pixelated.

b) regarding the original minor comment F) of this reviewer: pBAD is understood to be phosphorylated-BAD. When showing anti-pBAD western blots, usually anti-BAD western blots are expected. Reduced band intensity for pBAD should result from suppressed/blocked phosphorylation (or enhanced dephosphorylation). In a narrower sense, the molecule pBAD itself can not be "silenced" - "silencing" is from this reviewer´s experiencd primarily used in the context of gene expression.

c) Regarding to original minor comment G). This reviewer has clearly understood what the meaning of "notable expression pattern" is. I apologize for not clearly stating the concern. The intended question was: what exactly is notable about the expression pattern, please specifiy in which way the expression pattern distinguishes from the "not notable" expression pattern.

d) regarding original comment mjor H and minor K): this reviewer very much appreciates the immunofluorescence microscopy of cytochrome c release (also by FACS) and IF of activated (i.e. active) BAX! Thank you very much for the effort! Just for clarification: the subclone antibody 6A7 is specific for the N-terminur of BAX and therefore detects BAX exclusively in it´s active conformation (or denatured).

Reviewer #2

(Remarks to the Author)
The authors have done an excellent job on addressing my concerns, predominantly by the addition of a large amount of new data. I am now in favour of publication as is

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed all of my comments from my review of the initial manuscript. They have completed several further experiments and added to the text which significantly strengthens the manuscript. I agree that the manuscript can now be published.

REVIEWER COMMENTS (Point-by-point response)

Dear Reviewers,

We would like to extend our sincere thanks to each of you for your thorough review and thoughtful feedback on our study exploring the role of PIM2 in the mitochondrial control of programmed cell death in normal and tumor plasma cells. Building upon our 2022 study, which investigated the role of PIM2 in B cell precursors, our current work delves into the regulation of BCL2 family proteins within plasma cells, a unique and particular context of elevated PIM2 expression.

Over nearly two decades, we have honed our expertise in culturing primary human B cells and differentiating them in vitro into plasma cells. This experience has enabled us to develop robust techniques for protein exploration, including immunoprecipitation of endogenous proteins, with meticulous controls in place to ensure the reliability and accuracy of our results. While the expression of NOXA within the ISR pathway has been previously documented, our work reveals a novel role for PIM2 through its inhibition of ISR.

We appreciate the reviewers' detailed comments, which have raised important methodological and interpretive questions. In response, we have provided comprehensive answers in the following pages, supported by new experiments.
Specifically, we have further clarified the regulation of NOXA and confirmed that its induction under PIM inhibitor was independent of p53 in tumor plasma cells. Beyond NOXA, we also conducted additional investigations into the pro-apoptotic factor BIM using siRNA assays for deeper insights. We unraveled the distinct contributions of these 2 pro-apoptotic proteins in sensitizing cells to apoptosis under PIMi.
Furthermore, we have expanded our study to consider previous research on multiple myeloma cell lines expressing PIM1, PIM2, and PIM3. To delineate the unique role of PIM2 in plasma cell biology, we designed a new morpholino that specifically targets PIM3, and thus evaluated the effects of PIM3 knockdown in myeloma cell lines.

As noted by two of the reviewers, our work offers new perspectives on PIM kinases and underscores the therapeutic potential of targeting these kinases as part of a co-therapy for certain cancers.
We are grateful for the opportunity to enhance our manuscript and hope that our revisions satisfactorily address your concerns. Please find our point-by-point responses to your comments on the following pages.

Thank you once again for your valuable time and constructive feedback.

Sincerely,
Thierry Fest

Reviewer #1 - Apoptotic signaling, ISR:

Reviewer comments to the manuscript „Uncovering the Role of PIM2 in Modulating MCL1 Dependency and Activation of ISR-Mediated NOXA Expression“ by Marion Haas et al.

The manuscript presents substantial data regarding PIM2 inhibition/knock-down/out and its effect on Bcl-2 family regulated mitochondrial apoptosis signaling pathway. The authors claim that PIM2 inactivation alleviates BAD phosphorylation and activates integrated stress response thereby inducing expression of the pro-apoptotic BH3-only protein NOXA that inhibits MCL-1 resulting in increased (sensitivity to) apoptosis. The authors present a variety of convincing data covering a broad range of biological/biochemical aspects addressed by suitable basic (immunochemical, flow cytometric) and sophisticated methods. Finally, the authors present data to prove the ISR dependent pro-apoptotic (anti-tumoral) activity of combined PIM-inhibition and MCL-1 inhibition by co-applying the ISR-inhibitor ISRIB in a mouse model of femur injected myeloma cells. The results from mouse experiments are well in line (and even succeed) expectations from wet lab experiments.
Despite the overall very positive first impression this reviewer has a lengthy list of concerns, partially regarding essential aspects and data in the manuscript. These concerns are far beyond simple errors and must be addressed before considering publication in a highly renowned journal.

Major Points:

A) The title is misleading. Authors show the effect of PIM inhibition or knock-down – the results might originate from indirect and/or downstream effects.
Response:
Thank you for your thoughtful feedback regarding the title of our manuscript. We fully understand and appreciate your concerns about the potential for indirect or downstream effects resulting from PIM inhibition or knock-down.

Our study focuses on the specific inhibition of PIM kinases, particularly PIM2, using AZD 1208 and a morpholino approach. We meticulously examined the resulting modifications at the level of BCL2 family molecules, their binding, the threshold for apoptosis induction via MCL1 loading with BH3-only molecules, and ultimately, the induction of the ISR pathway through NOXA production. These effects, as demonstrated in our study, are directly linked to the inhibition of PIM2 in plasma cells, leading us to conclude that PIM2 plays a crucial role in controlling, influencing, and functionally modifying MCL1 dependence and the activation of the ISR pathway.

Given this, we believe our title is both clear and reflective of the key messages and findings of our work. It aligns with the content of the paper and effectively communicates our central findings to the reader. Moreover, we took care to specify in the Introduction that our investigation was centered on inhibiting PIM kinase activity.

We also note that the other two reviewers did not express concerns regarding the title, suggesting that it meets the general expectations for clarity and accuracy in the field. For these reasons, we have decided to retain the title as it currently stands.

Thank you once again for your valuable input.

B) The principle finding that ISR induces NOXA expression has already been published (Cell Death Discovery (2022) 8:215).
Response:
We fully agree with the reviewer that NOXA expression downstream of the ISR pathway has been described previously. Our point is that PIM2 inhibition induces NOXA in ASCs, which is new. In the manuscript, lines 219-220, we stated the fact that NOXA production may be due to ISR activation, we added two references (PMID:29352505; from 2018 and PMID:32648994; from 2021). To complete our references, we propose to add the year 2022 reference suggested by the reviewer in Cell Death Discovery, which paper reported NOXA production after ISR induction and also referenced PMID:29352505 on this point.

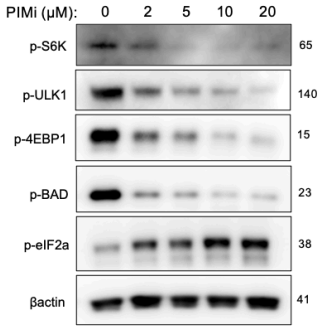
C) Co-IP experiments look impressively clean (no detection of heavy or light chain) and efficient. Lysates from knock-out or over-expressing cells would serve as additional control. This is absolutely important when analyzing interaction and activity of Bcl-2 family proteins since authors use NP-40/deoxycholate as detergents (line 763). Conformational change of BAX and interaction with BCL-xL, Bcl-2 has been shown to be induced by non-ionic detergents (J Biol Chem (1997) 272:13829-34). This might also apply for the interaction of other anti-apoptotic and BH3-only proteins.
Response:
Thank you for your positive feedback on the cleanliness and quality of our co-IP data. We take great care in our protocols and use of primary cells to ensure high-quality results.
Regarding the reviewer's comment on BAX and its binding properties in relation to the detergent used in our protein extraction buffer, we are well aware of the work by Richard Youle's group. BAX is a member of the executioner group of the BCL-2 family and, along with BAK, contains all four BH domains. Both BAX and BAK, upon activation, form pores in the outer mitochondrial membrane. In their inactive state, BAX and BAK adopt a typical BCL-2 fold, but unlike the guardian proteins (such as BCL-2 and BCL-XL), whose folds are stable and form high-affinity complexes with BCL-2 family partners, BAX and BAK undergo significant conformational changes during activation that lead to oligomerization and pore formation. There are notable differences between BAX and BAK, including tissue distribution and subcellular localization prior to activation. BAX primarily resides in the cytosol, whereas BAK localizes to the mitochondrial membrane. The studies by Hsu and Youle in the late 1990s (PMID: 9153240 and PMID: 9553144), cited by the reviewer, demonstrated that cytosolic BAX in murine thymocytes can undergo detergent-induced conformational changes, leading to homodimer or heterodimer formation with BCL-2 and BCL-XL. However, once activated, BAX and BAK undergo further conformational changes and relocalize to the mitochondrial membrane, where the membrane environment plays a crucial role in regulating their interactions.
Interestingly, Youle's group demonstrated in 2011 that the complex between the guardian BCL-XL and the executioner BAX involves clear binding at the membrane, but without accumulation at the mitochondrial outer membrane (MOM). Instead, BAX interaction with BCL-XL leads to its 'retrotranslocation' into the cytosol, where the complexes disassemble (PMID: 21458670). This finding underscores the complexity of BAX biology, particularly its subcellular localization. Additionally, in the late 1990s, Youle's group challenged the prevailing dogma that BAX homodimers promote cell death, and that the formation of BAX homodimers is prevented by BAX

heterodimerization with prosurvival factors BCL-2 and BCL-XL (PMID: 8358790, PMID: 7834748).
It is important to note that these detergent-induced conformational changes have been specifically observed in cytosolic BAX, and not in other BCL-2 family members, including MCL1, BCL-XL, and BAD, which are the focus of our study (Figures 4 and 5). Additionally, many recent studies have emphasized the significant role of the membrane environment in modulating interactions among BCL-2 family proteins, often using detergents to solubilize membranes (PMID: 37438560).
Furthermore, our experimental results, which show specific modulation of protein binding in response to PIM or MCL1 inhibitors and ISRIB treatment, argue against any significant impact of detergent use on the binding interactions we observed.

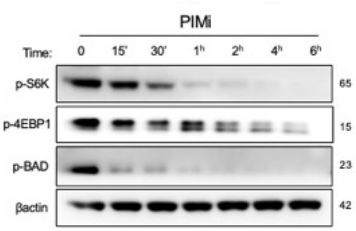
Given these considerations, we believe that investigating potential binding modifications of MCL1, BCL-XL, and BAD due to the use of NP-40/deoxycholate is not pertinent to our study. Such an exploration would require extensive additional work without a clear benefit based on the current understanding of BCL-2 family protein interactions.

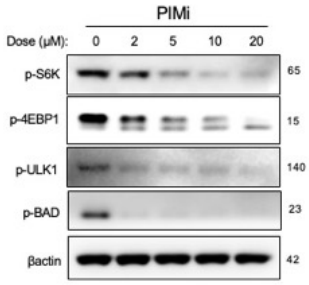
D) The author´s present a huge set of data. However, there is almost no re-production of published characteristics making it hard to judge whether the investigated aspects are solely responsible for the presented results. Specifically: what happens to cells incubated with PIMi (AZD1208) regarding mTOR (e.g. Blood (2013) 122: 1610–20.), autophagy, cell cycle (e.g. Cancer Res (2008) 68: 5076–85)? At least some of these aspects need to be included to estimate the importance of pBAD, ISR, NOXA in relation.
Response:
Our study involved a large amount of data and experiments to establish and demonstrate this novel role for PIM2 in stress modulation in plasma cells.
PIM kinases have been described as being involved in many biological processes, often without really distinguishing between the three PIM kinases for a given cell. As indicated by the reviewer, there is some evidence for changes in the mTOR pathway following inhibition of PIMs by AZD1208. As requested by the reviewer, we investigated the impact of PIM inhibition on the mTOR pathway in primary plasmablast cells obtained at day 6 of in vitro culture (panel A, below). Cells were treated at different doses of AZD1208 for 6 hours, showing a gradual inhibition of the mTORC1 pathway as assessed by phosho-S6K (p-S6K) expression. In parallel, we detected a common decrease in protein translation through reduced expression of phospho-4EBP1 (p-4EBP1), as well as autophagy with reduced phospho-ULK1 (p-ULK1). As expected, these results are accompanied by a decrease in p-BAD, while p-eIF2a increases, as described in the manuscript. These results were verified in the multiple myeloma LP1 cell line either by time-lapse exposure to a fixed dose of 20uM AZD1208 or by testing AZD1208 escalation after 6 hours of treatment (panels B, below).

Panel A



Panel B





Thank you for your suggestion. After careful consideration, we have decided not to include these results in the revised manuscript, as we believe they fall outside the primary focus of our study.

E) Concerning the differentiation model of B-cells in Figure 1: the authors have any suggestion why NOXA is only significantly upregulated in D4 aBCs and not at later stages of differentiation? Are dead cells increasing during differentiation that are harvested?

Response:

NOXA expression increases in our B cells after four days of activation following the first cytokine cocktail, which induces a proliferation "boost" as described in a previous study (Pignarre et al. 2021, PMID: 33150420). In this study, we did not investigate this increase in NOXA at this stage of B cell maturation.

However, in a previous paper (Santamaria et al. PMID: 33995412) we described that these cells already show signs of mTORC1 and UPR activation, especially in the most proliferated cells. Day 4 activated B cells are therefore subject to cellular stress which is certainly responsible for the induction of NOXA (by the ISR pathway), but not compensated by a sufficiently strong increase in PIM2 kinase. This is not the case in generated plasmablasts, in which NOXA expression has almost completely disappeared, because the cellular stress induced is not capable of inducing ISR due to the high expression of PIM2. The high stress in plasmablasts/plasma cells is nevertheless characterized by a strong increase in BIM (Fig. 1b in the manuscript). Thus, we can speculate that NOXA, and more generally ISR, is not inhibited at this day 4 stage of differentiation, since PIM2 expression in these cells is not sufficient to counteract ISR induction, either for reasons of quantity and/or for other direct or indirect post-translational reasons, such as its compartmentalization within the cell.

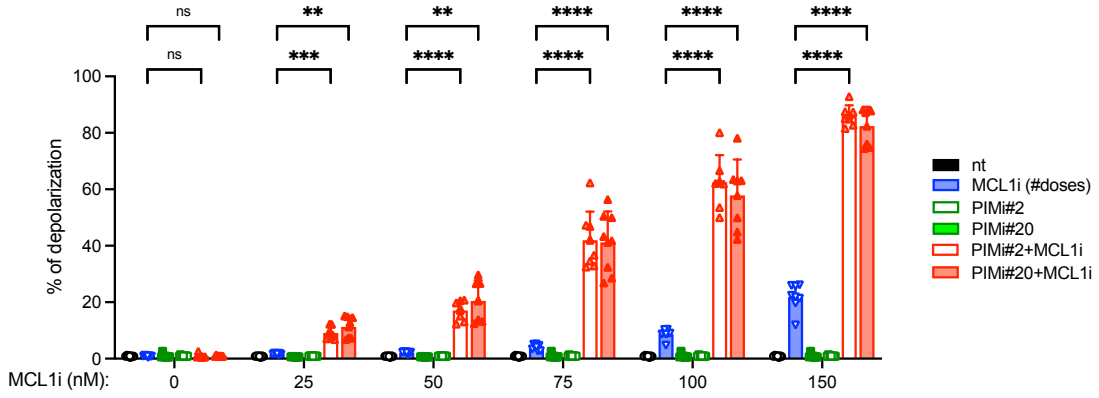
F) The inhibitor AZD1208 has been published as pan-PIM inhibitor. Concentrations of AZD1208 in a number of experiments is rather high (μM) – the authors need to exclude off-target effects more rigorously, e.g. by applying the inhibitor to PIM2-knock-out cells.

Response:

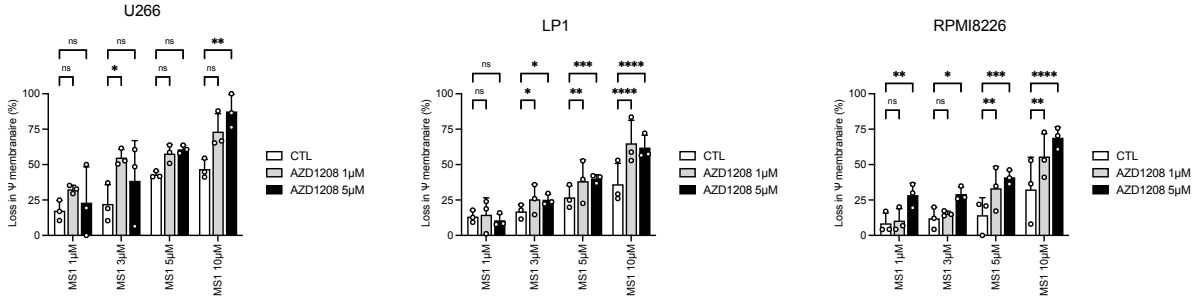
The doses of AZD1208 used in our study are higher than those typically used in other cancer cells due to the significantly elevated levels of PIM2 in plasma cells compared to other cancer types. For our functional experiments—such as the characterization of the ISR pathway and the phosphorylation of BAD in immunoblotting—we employed short PIM inhibition times, necessitating higher doses of the PIM inhibitor (PIMi) to observe effects. However, with longer incubation periods, lower doses can achieve the same outcomes. Moreover, as shown in Supplementary Fig. 5a, our data indicate that

even at low doses, AZD1208 exhibits significant synergy when combined with AZD5991(MCL1 inhibitor).
To directly address the reviewer's question regarding the consistency of the synergy between pan-PIM and MCL1 inhibitors at a tenfold lower dose of AZD1208, we conducted additional experiments using the XG7 MMCL. We measured mitochondrial membrane depolarization with increasing doses of AZD5991 in the presence of either 20 µM (as presented in the manuscript) or a 10-fold lower dose (2 µM) of AZD1208. The results are clear: while AZD1208 alone does not induce depolarization at either dose, synergy between the two drugs was observed at the lower dose of 2 µM AZD1208, with efficacy comparable to that at 20 µM (Panel A). We further assessed the response using BH3 profiling with reduced doses of the PIM inhibitor. In RPMI8226, LP1, and U266 cells, we tested increasing doses of the MS1 peptide and confirmed the increased MCL1 dependency following PIM kinase inhibition, whether using 1 µM or 5 µM of AZD1208 (Panel B). These results are consistent with those obtained using 20 µM of AZD1208 in our previous experiments.

Panel A



Panel B

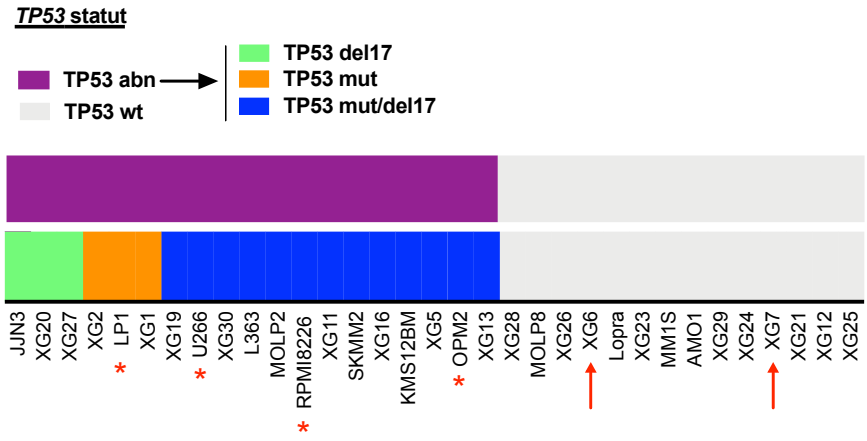


The results in question have not been included in the revised version of the manuscript because we believe they are outside the scope of our study.

G) The rational for selecting the indicated cell lines is unclear. Some of the cell lines are not standard and information on mutational status of important proteins is lacking. Since NOXA has been originally published as p53 target, at least p53 mutational status of the investigated cell lines must be shown – especially since the authors conclude that p53 expression is not affected by PIMi (which indicates mutation of p53)!
Response:

We have had a long-standing collaboration with Jérôme Moreaux and his team at the IGH, CNRS in Montpellier, France. Over the years, this team has established several multiple myeloma cell lines (MMCLs), many of which have been molecularly characterized through exome sequencing, with the results published in 2019 (PMID: 30809292). In Figure 5B of that publication, *TP53* mutations in various cell lines were documented. Of the six cell lines (MCL1-dependent) used in our study, RPMI8226, OPM2, LP1, and U266 were identified as *TP53*-mutated, while XG6 and XG7 were confirmed as *TP53* wild-type (panel A, below).

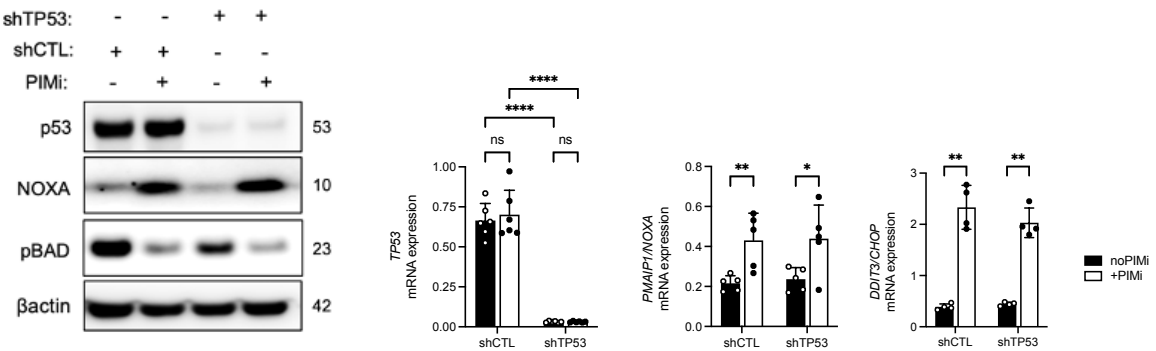
Panel A



These results have been included in the revised version of our manuscript on Fig. 3d.

Recognizing the importance of the independence of NOXA expression from the p53 transcription factor in the context of myeloma, as highlighted by the reviewer, we have generated new data to address this issue. With the assistance of Moreaux's team, we transduced the XG7 line (wild type for *TP53*) with shRNA to knock down p53 protein expression (shTP53). We then compared these p53-deleted cells to their wild-type counterparts transfected with a mocked shRNA (shCTL), and specifically examining NOXA induction following AZD1208 treatment. Our results showed that the absence of p53 had no effect on the induction of the ISR as measured by *DDIT3*/CHOP and *PMAIP1*/NOXA mRNA expression and especially on NOXA protein production (panel B, below). These findings conclusively demonstrate that NOXA expression induced by AZD1208 is not mediated by p53 but is instead a direct result of disrupted PIM kinase activity.

Panel B



These results have been included in the revised version of our manuscript on Fig. 3e (Lines 212-218). We have also added the reference (PMID: 30809292) to the bibliography, and Jérôme Moreaux has been included as a coauthor of the paper.

H) Is it correct, that the percentage of Casp-3 positive cells in Figure 2F/3G is 1% - 3 %? Or is this shown relative to the control?
Response:
The reviewer is right; we made an error in the initial submission. The results presented the ratio of % of Casp3a positive cells of the tested condition to the % of Casp3a positive cells of the control condition. We corrected the legend of each plot accordingly

We appreciate the reviewer's observation and have updated the figure legends accordingly for Fig. 2f, 2g, 2h and Fig. 3i in the revised manuscript.

In the methods part the authors state that for Caspase-3 active and TMRM staining (flow cytometry) dead cells were excluded from the detection via DAPI staining (Lane 742). In the same regard, in lane 123 and 124, the authors talking about "full caspase 3 activation". This might be misleading if caspase-3 activation was assessed in viable cells. The authors should clarify how to interpret these data.
Does that mean that the data in Figure 2F/3G show that less then 5 % viable cells are positive for caspase-3?
Response:
The reviewer is right, once again; we made an error in the initial submission. The results presented were based on the ratio of the test condition to the control condition and we found 5 times more Casp3+ cells in tested condition than in the control. In addition, see response to the next question…

We appreciate the reviewer's observation and have updated the figure legends of Fig. 2f and Fig. 3i (formerly 3G) accordingly in the revised manuscript.

Why were dead cells excluded in the first place for assessment of apoptosis after PIMi? Same is true for the TMRM staining, which would then only reflect the shift in membrane potential for viable and not dead cells.
Response:
Our cytometric gating strategy is based on the behavior of primary cells during stage two of in vitro differentiation (Pignarre et al., PMID: 33150420). By day 7 of culture, 40-50% of B cells have died in the absence of CD40L, BCR, and CpG stimulation. Only certain post-activated B cells and plasma cell progenitors survive, primarily due to the upregulation of anti-apoptotic factors like PIM2 (Haas et al. 2022, PMID: 35108359). Therefore, when working with in vitro generated primary ASCs on day 7, we systematically exclude DAPI+ cells, as these are completely dead because of the physiological process of the differentiation. We focus our analysis on DAPI- cells to assess the response of alive cells to engage apoptotic process after 6 hours treatment by PIMi through caspase3-active labeling. It is important to note that after 6 hours treatment by PIMi, we did not induce DAPI+ cells, only DAPI/Casp3a+ cells have been induced. We made this gating strategy to exclude no specific dead cell.

I) Figure 4C/D/E/I: PIMi seems to downregulate MCL-1 or increase its degradation (at least in RPMI8226/U266 cells). However, this is not considered in the schematic model in 4I. Assumingly, the reduced levels of MCL-1 also contribute to the increased

apoptosis response? Also, was the reduced level of MCL-1 considered for the subsequent co-IPs showing that MCL-1 is binding more BIM/NOXA (Figure 4G). If not, it remains unclear whether the increased MCL-1 binding results from reduced MCL-1 abundance in these cells. At the very least, the authors should include a discussion of these critical points.

Response:

The biology of MCL1 remains complex and not fully understood. In our study of MMCLs, we identified RPMI8226 and U266 as two of six cell lines showing a significant decrease in MCL1 protein levels following 24 hours of high dose of AZD1208 treatment, parallel to the increase of NOXA expression (Fig. 4c). As previously reported in reference #17, NOXA binding to MCL1 can promote its proteasome-dependant degradation. This prompted us to further investigate the observed decrease in MCL1 under AZD1208 treatment (Figure 4d, e and Supplementary Fig. 4a, e).

Our experiments using MG132, QVD-OPH, and cycloheximide demonstrate that PIM inhibition by AZD1208 induces a decrease in MCL1 that is proteasome-independent but instead occurs after the activation of apoptosis and caspase activity. This aligns with reference #16, which describes MCL1 degradation by caspases.

Accordingly, we have made slight modifications to the relevant text in the revised manuscript (Lines 310-317).

Regarding the reviewer's question about whether the decrease in MCL1 protein after AZD1208 treatment should be considered in our co-IP experiments, we believe this is not a concern. As shown in our immunoblots (initial version of the manuscript, Figure 4G), MCL1 levels in both the input and IP samples remain consistent (see the last band of the blot), indicating that the amount of MCL1 protein immunoprecipitated is unchanged.

Moreover, the pre-treatment duration with PIMi for our IPs was less than 24 hours, which minimizes the likelihood of significant MCL1 degradation. Additionally, Caspase 3 induction occurs rapidly after the addition of MCL1i (AZD5991). To prevent excessive caspase-dependent protein degradation, we optimized the doses and incubation times of MCL1i to achieve a controlled level of Caspase 3 activation, around 20%. This level is sufficient to observe effects without causing extensive degradation. This approach has been validated by other studies conducting similar experiments (PMID: 36346691).

Regarding the comment on modifying the cartoon in Figure 4I, we prefer not to make changes. The model we propose accurately summarizes the changes induced shortly after AZD1208 treatment, occurring well upstream of caspase activation and their likely role in the degradation of proteins, including MCL1.

Please note that we discovered an error in the manuscript submitted to the reviewers: line 244 mistakenly refers to " Figure 4C " instead of " Supplementary Fig. 4c". We have corrected this in the revised version.

J) Western blots are in part inconsistent regarding expression of central players in apoptosis regulation, most importantly downstream effectors BAX and BAK. In this regard critical point regards to comparably low expression of BAX and BAK in RPMI8226 – how does this fit to apoptosis induction via mitochondrial pathway?

Response:

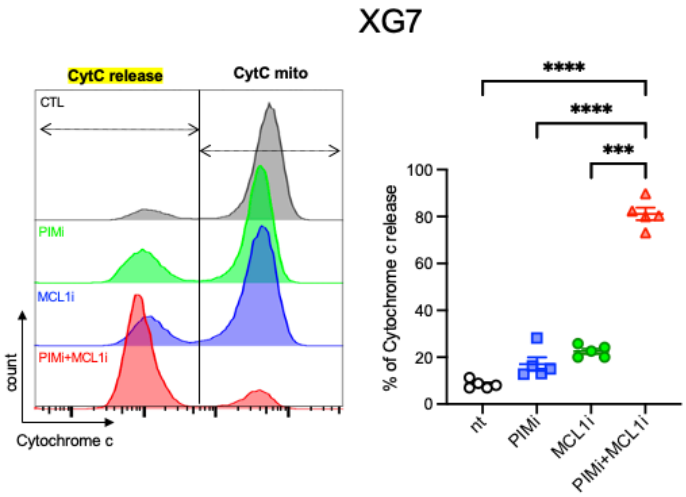
The expression levels of BAX and BAK vary depending on the cell lines examined. Some tumor cells do not express BAX due to mutations that may directly cause a

defect in MOMP depolarization. None of the MMCLs studied in our paper showed such an abnormality (PMID: 30809292). Therefore, we believe that the level of BAX and BAK expression in RPMI2886 cells is not representative of the ability of these molecules to participate in the execution of apoptosis. Furthermore, it has been described that multiple myeloma cells are highly dependent on the absence of BAK activation for their survival (PMID: 32371863 & PMID: 30309889).

Authors should show release of cytochrome c in addition to TMRM.
Response:
As requested by the reviewer, we conducted experiments to assess cytochrome c release following the same protocol as for TMRM experiments. Detection was performed by flow cytometry on the XG7 cell line, comparing different conditions, including the combination of the pan-PIM inhibitor AZD1208 and the anti-MCL1 inhibitor AZD5991. This drug combination clearly resulted in the highest level of cytometric cytochrome c release (see panel A below).

This result has been incorporated into the Supplementary Fig. 5j of the revised manuscript (Lines 377-379) and we added the protocol for the cytochrome c release in the methodology section.

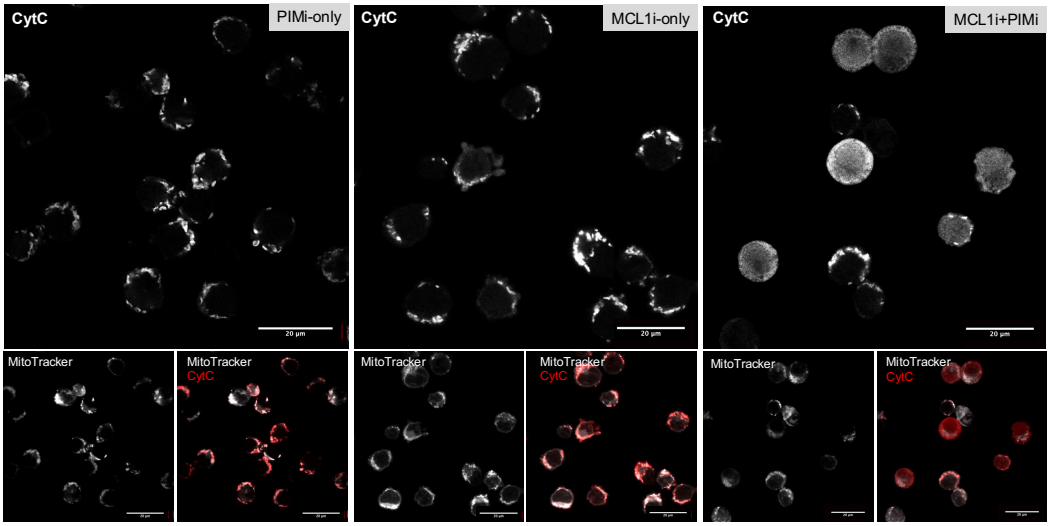
Panel A



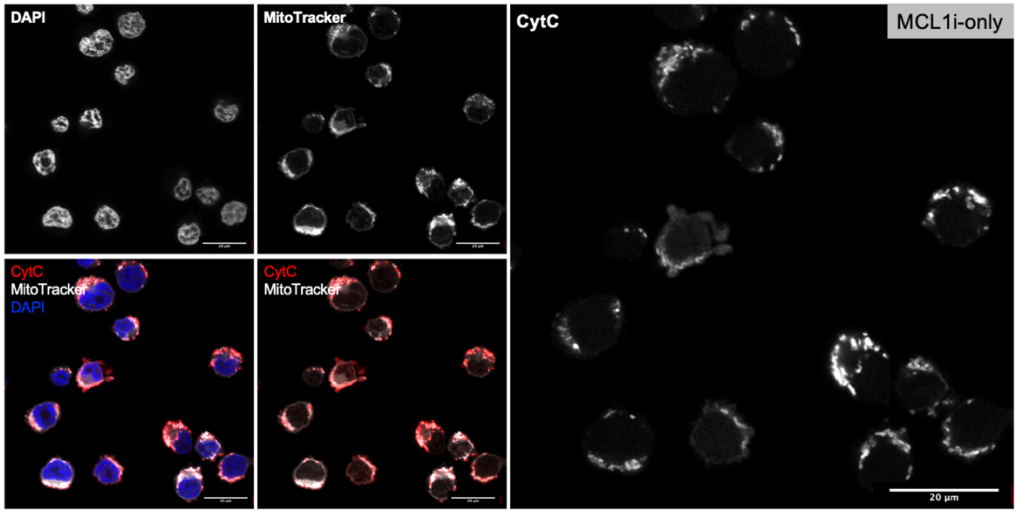
Additionally, in response to the reviewer's comment 'L' below, we also performed immunofluorescence labeling of cytochrome c; mitochondria were identified with the MitoTracker dye. Our results show a diffuse Cytochrome c labeling with the drug combination compared to the inhibitor alone, where cytochrome c is confined within the mitochondria. These data confirmed the release of Cytochrome c, which spreads throughout the cell upon dual drug treatment.

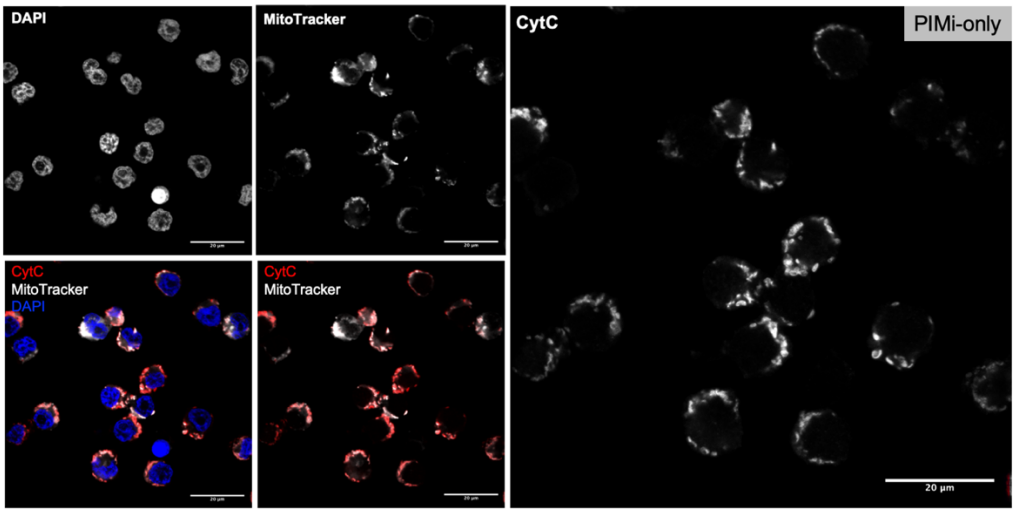
In the revised manuscript, we have included pictures summarizing these results in Fig. 5e (see below Panel B) and Supplementary Fig. 5l (see panel C below).

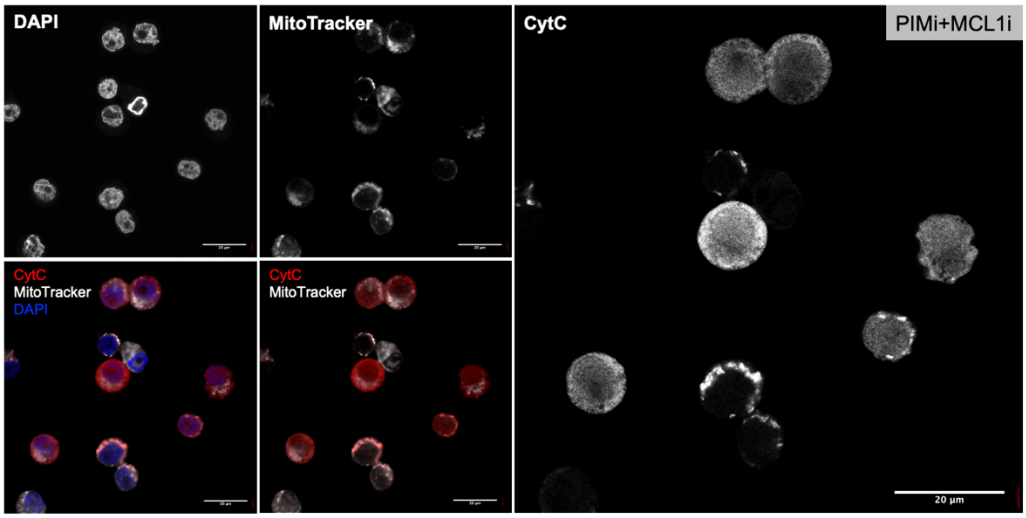
Panel B



Panel C







In this regard: what do the authors want to indicate with “right shift of TMRM signal = mitochondrial depolarization” (Fig5AD, supplFIG5EG – missing in supplFIG5F). This reviewer expects a left-shift due to depolarization (loss of deltaPSIm, reduced fluorescence).

Response:

Once again, we thank the reviewer for their careful attention. You are correct that the shift observed is a 'left-shift,' not a 'right-shift,' as previously stated.

We have corrected this error in Fig. 5a and Supplementary Fig. 5f, n, o and have also made the necessary updates to Supplementary Fig. 5h.

K) What is the rational to switch to another cell line (XG7)?

Response:

Why use XG7? There are several reasons for this choice. When selecting cell lines for our study, we aimed to use the one that would best demonstrate the involvement of the ISR pathway in NOXA production. In vitro, XG7 exhibited the strongest synergy between the two drugs, PIMi and MCL1i, making it an ideal candidate for further study in mouse xenografts. In contrast, although U266 cells show some induction of NOXA with AZD1208 treatment, the overall expression is low and variable, reducing their relevance for our research. Additionally, U266 cells are particularly resistant to drug combinations, further limiting their utility. Finally, we chose not to use the RPMI8226 cell line due to its inherently high levels of NOXA expression, which we felt would not be an appropriate selection criterion for our study.

L) Subcellular localization of key proteins (e.g. BAX, MCL-1, cyt c) should be investigated, e.g. by immunofluorescence microscopy.

Response:

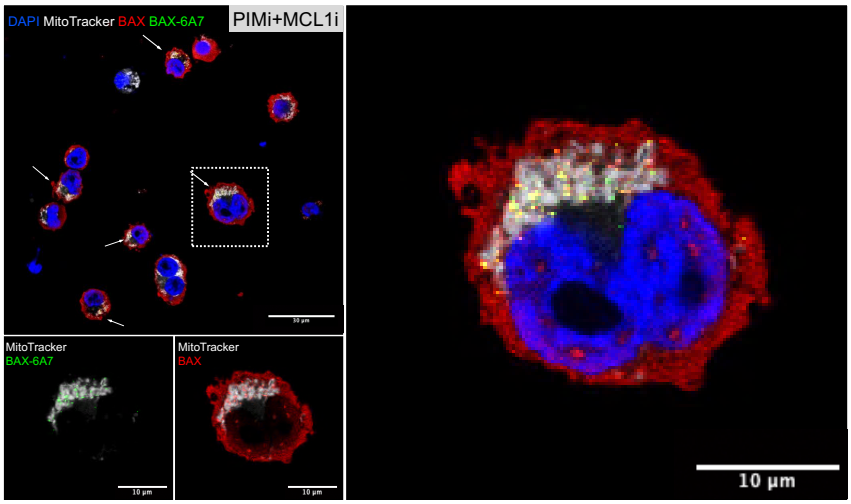
As requested by the reviewer, we conducted immunofluorescence experiments to illustrate the induction of mitochondrial depolarization and subcellular localization of key proteins as BAX and Cytochrome c.

After to have evaluated Cytochrome c release by flow cytometry, we observed a high diffusion of Cytochrome c by immunofluorescence microscopy, for the combined inhibitor condition.

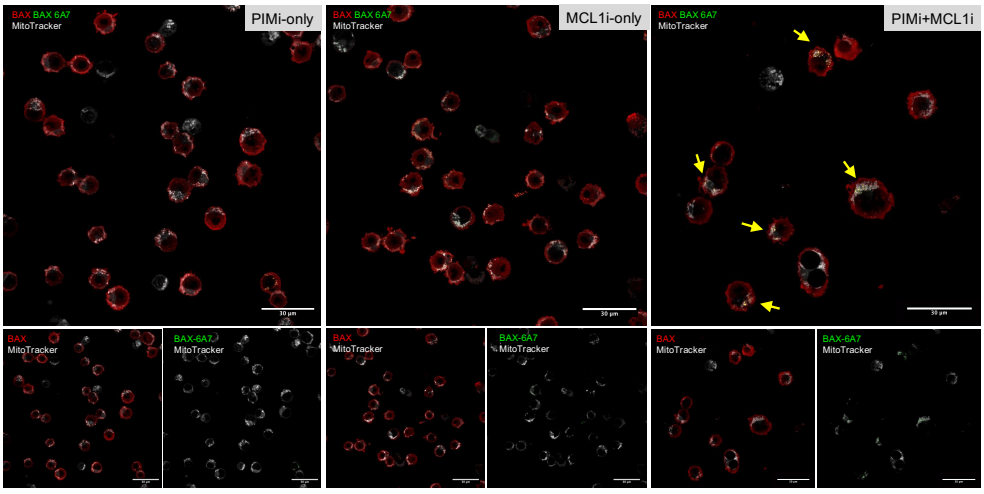
To further our investigations on subcellular localization and as requested by the reviewer, we visualized the expression of BAX, BAX total and the active form of BAX (using the BAX 6A7 antibody), to complement Fig. 5d of the manuscript. This antibody specifically detects the active forms of BAX that associate with mitochondria during membrane depolarization and subsequent cytochrome c release.
Our results show – as expected and describe in our response to comment “c” - that BAX has a diffuse localization in XG7 cells in all condition, but we observed some complexes of protein in the mitochondria which colocalized with BAX 6A7 labeling in the condition combining both drugs (indicated by the white arrow). The other three conditions exhibit only rare BAX 6A7-positive cells at the mitochondria.

In the revised version of the manuscript, Fig. 5d presents a depolarized cell who BAX 6A7 (green) colocalized with MitoTracker dye (white), while full-length BAX protein (red) is broadly distributed throughout the cytoplasm (panel A, below). The revised manuscript has been modified accordingly (Lines 396-404).
Additional images under different conditions are provided in Supplementary Fig. 5i (panel B, below).

Panel A



Panel B



M) Fig2B: how does marginal induction of NOXA expression fit to highly successful mouse model results? Does apoptosis of U266 cells really rely on NOXA induction? Fig2E: 20 µM – is the inhibitor still specific at this concentration?
Response:
We agree with the reviewer regarding the relatively low induction of NOXA under AZD1208 treatment in Fig. 2b for U266 cells. However, if we look at Fig. 2e with U266 cell line, which is an independent experiment, the increase in NOXA is much more pronounced. Additionally, in the co-IP results shown in Fig. 5b, we observe a clear increase in NOXA protein bound to MCL1 in U266 cells.
These observations highlight that variability can occur between experiments, particularly in the case of U266 cells and NOXA induction under AZD1208 treatment. As mentioned in our previous response to comment "k", this variability was one of the reasons we chose to focus on XG7 rather than U266 for our work on ISRIB.
Overall, we remain confident in the results obtained with U266 cells, as they are consistent with findings from primary plasmablasts and other MMCLs. We believe that NOXA is a key factor in the response to AZD1208 for U266, which also explains the in vivo results observed in mice.
Regarding reviewer's comment about the specificity of the response in relation to the doses used, we have addressed this question above in a previous response.

N) CRISPR/Cas9-screen is not explained in the methods part – please correct
Response:
The CRISPR/Cas9 screening of myeloma cell lines is not included in the methods because, as indicated in the text, these results come from the DepMap Portal project, where all methodological and technical details are provided (https://depmap.org/portal/).
The revised version of the manuscript, we added details concerning this data (Lines 70-71).

O) Staining procedure for FACS analysis (Fig6E, 7D) is not included – please correct
Response:
We apologize for the oversight and have made the necessary corrections in the revised version of the manuscript.
Gating strategy of Fig. 6e are presented in Suppl Fig 6d and those of Fig 7d in Suppl Fig 7b.

P) Fig4B: concentration of BH3-peptide is missing – please correct
Response:
We apologize for the oversight and have made the necessary corrections directly in x axis legend of graphs in Fig. 4a, b.

Minor points
A) Line62-63: rephrase
Response:
We have modified the sentence (lines 64-65 of the revised version).

B) Line 65: explain CRISPR/Cas9 data
Response:

Please see our above response concerning data obtained from the DepMap Portal project and the modification in the manuscript (lines 70-71).

C) Lines 66, 68: rephrase precisely “levels”. Take care about gene and protein names.
Response:
Done, please see lines, 75 and 76-77.

D) Fig1E and following: order of cell lines differs from supplFIG1C and chages in following figures – this is somewhat confusing. NOXA expression is missing for U266 – please comment.
Response:
I apologize for the differing order in some of the figures; the reviewer will understand that it is not physically possible to rearrange the blots.
Regarding NOXA in U266, I assume the reviewer is referring to Figure 2B and the observed low NOXA production after AZD1208 treatment. This issue has already been addressed in a previous response to major comment “m”.

E) Fig1F: it would be awesome to include relative protein expression level in the table.
Response:
Thank you for your comment. We feel that Western blot results are quite easy to interpret and see the difference, we prefer not to add more details in a paper that already has a “huge” number of data, as the reviewer noted in major comment "d".

F) Line 100: what is “pBAD silencing” supposed to mean. Please rephrase.
Response:
Is “suppression” better? Line 113 we have modified it accordingly.

G) Line 0: what is “notable expression pattern”? – explain.
Response:
Line 115, means a “remarkable or intriguing” expression profile during B-cell differentiation.

H) Fig3, micrco abbreviated with “u” instead of Greek letter.
Response:
Ok, we’ve modified the Figure accordingly

I) Fig3G: indicate statistical test.
Response:
OK, it’s done

J) Fig4 labelling: RPM8266 vs previously RPMI8226
Response:
Ok, we’ve corrected the labelling accordingly

K) Fig4I: the authors do not show data on subcellular localization. Schematic is not supported by data. Apoptotic threshold has been published decades ago.
Response:
As requested by the reviewer, we have included microscopic images to illustrate the effect of the PIMi and MCL1i combination on subcellular localization of key proteins as BAX (whose active form BAX 6A7 has been found exclusively in the mitochondria and

specifically under the combinaison treatment) and Cytochrome c (initially localized in the mitochondria, and which are release following the depolarization and diffuses in cells).
The cartoon in Fig. 4i is intended to provide a concise summary of the various results presented in our study. While it may not capture every detail perfectly, it offers valuable insight and aids in the reader's understanding, which is particularly important for a journal like *Nature Communications* that targets a broad scientific audience.

L) Fig5B: MCL-1 inhibitor increases expression level of MCL-1 in U266.
Response:
It has been previously reported that treatment with a specific MCL1 inhibitor, such as AZD5991, results in increased detection of MCL1 protein. AZD5991 stabilizes MCL1 protein expression, a phenomenon widely described in the literature across various cell lines and particularly in PMID: 36346691. In our study, since U266 cells are not particularly sensitive to AZD5991, we used higher doses and longer incubation times. This likely explains why the increase in MCL1 protein in the input is especially pronounced after AZD5991 treatment.

M) Fig5D: this reviewer is well aware that WB analyses are not quantitative measurements. However, band intensities for BAX and BAK in Fig5D differ significantly from Fig1.
Response:
Yes, there are differences, which are simply due to the varying exposure times used to reveal our Western blots. Depending on the proteins being analyzed and the antibodies used, we adjust the exposure times to ensure consistency for each experiment. We believe the reviewer will understand and agree with this methodological choice.

N) Fig5DF – “right shift” – see above
Response:
OK, we’ve corrected the figure accordingly.

O) Fig5G: concentration of MCL-1inhibitor missing
Response:
OK, we’ve corrected the figure accordingly.

P) Figure 6/7: authors should state in the legend what kind of image is shown in the insert (brightfield?, only written once into the image in Figure 7E). Scale bars are missing
Response:
We have added scales to the figures. The inset images are provided as white light overlays to give an overall impression; Brightfield term has been added to the insets. We have also updated the figure legends and the 'Materials and Methods' section to reflect these changes.

Q) Fig7: switching to “two inhibitors” is unfortunate and inconsistent. Please use consistent labeling
Response:
We have added a footnote to the figure legend

R) supplFig3C: explain statistics. Statistical significance seems unlikely.

Response:
We've modified the image by simplifying the representation and indicating the statistics in the revised manuscript in Supplementary Fig. 3d.

S) supplFig5E(F)G: see above “right shift”.
Response:
OK, we’ve corrected the figure accordingly.

T) supplFig7F: “Enrichssement” score – please correct. Text in turquoise and light green is almost invisible.
Response:
OK, we’ve corrected the figure accordingly.

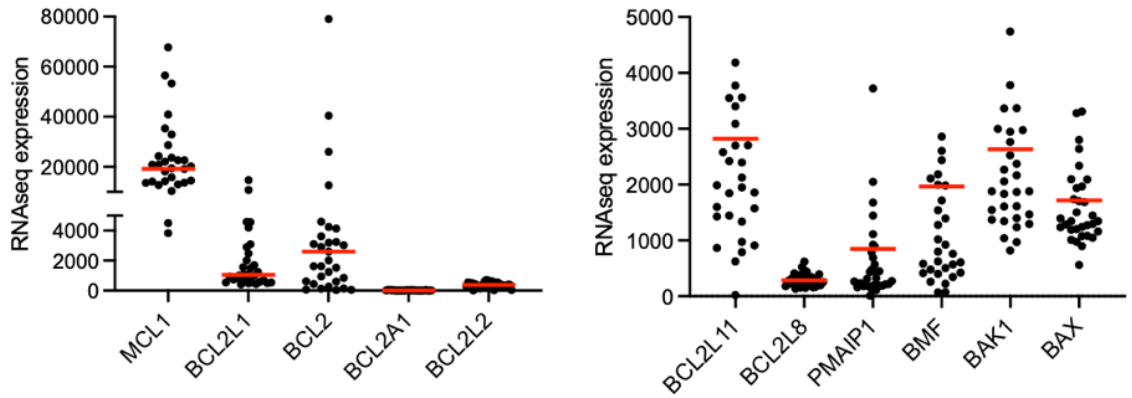
Reviewer #2 - MM therapy (Remarks to the Author):

The study of Haas and colleagues examines the functions of PIM2 in mediating the survival of normal and malignant human plasma cells. They use inhibitors and genetic knockdown approaches to define Pim2 interactions with the mitochondrial survival proteins and the integrated stress response, key drug targets in multiple myeloma. They show strong synergy between PIM and MCL1 inhibitors both biochemical assays and in vivo xenograft models for multiple myeloma.
Overall, this is a very thorough and robust study with many interesting aspects. The data is generally clear, and the authors tackle the problem with several orthogonal approaches that all lead to similar conclusions. I feel this excellent manuscript would be suitable for Nature Communications after some relatively minor changes.

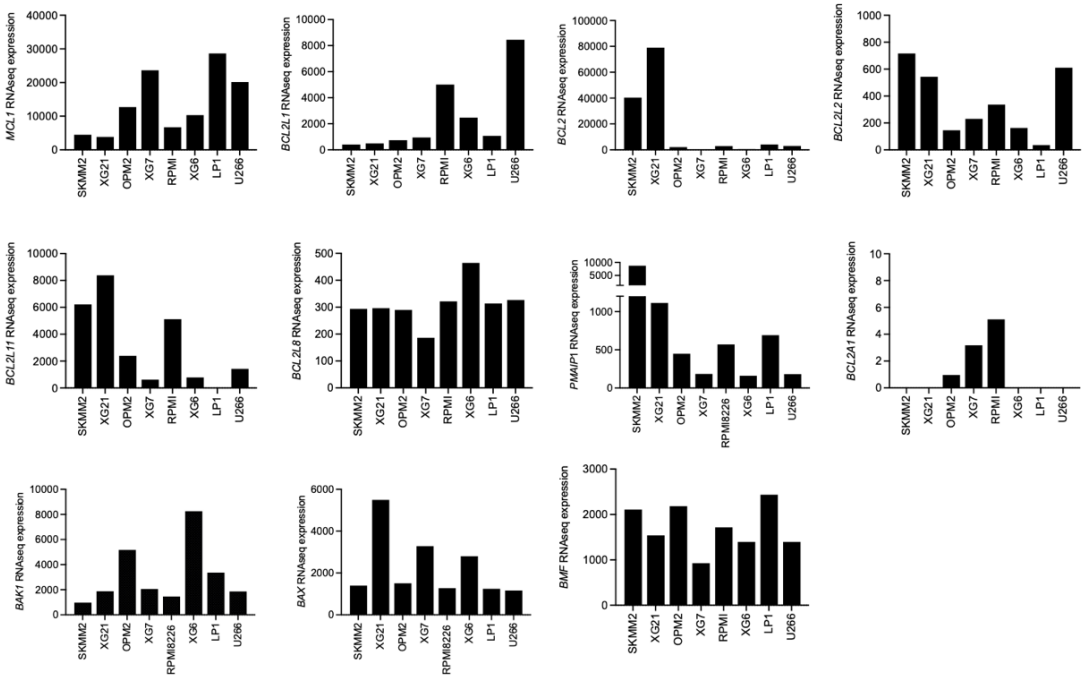
Specific comments.

1. Figure 1. This data is used to set the stage for the study by defining the expression of key pro/anti-apoptotic genes in normal and malignant human plasma cells. For completeness it would be useful to also include data for BFL1/A1 and BMF (at least at the RNA level) as these genes may also be relevant players for the models proposed.
Response:
Using Jérôme Moreaux's RNAseq data (now coauthor of the paper), we explored the RNA expression levels of BFL1/A1 and BMF. We also included data for BCL2L2.
These findings are presented below and have been incorporated into the revised manuscript as Supplementary Fig. 1e, f (Panel A, and B below). In Panel A (Suppl Fig. 1e), we display data from 33 multiple myeloma cell lines (MMCLs). On the left, we compare the expression levels of anti-apoptotic factors, and on the right, those of pro-apoptotic molecules. Notably, *BCL2A1* and *BCL2L2* genes are expressed at very low levels in MMCLs, especially compared to *BCL2L1* and *BCL2*, while *BMF* shows a relatively high level of expression, comparable to other molecules. In Panel B (Suppl Fig. 1f), we present the expression levels of these factors for each of the 8 cell lines of interest in our manuscript.

Panel A



Panel B

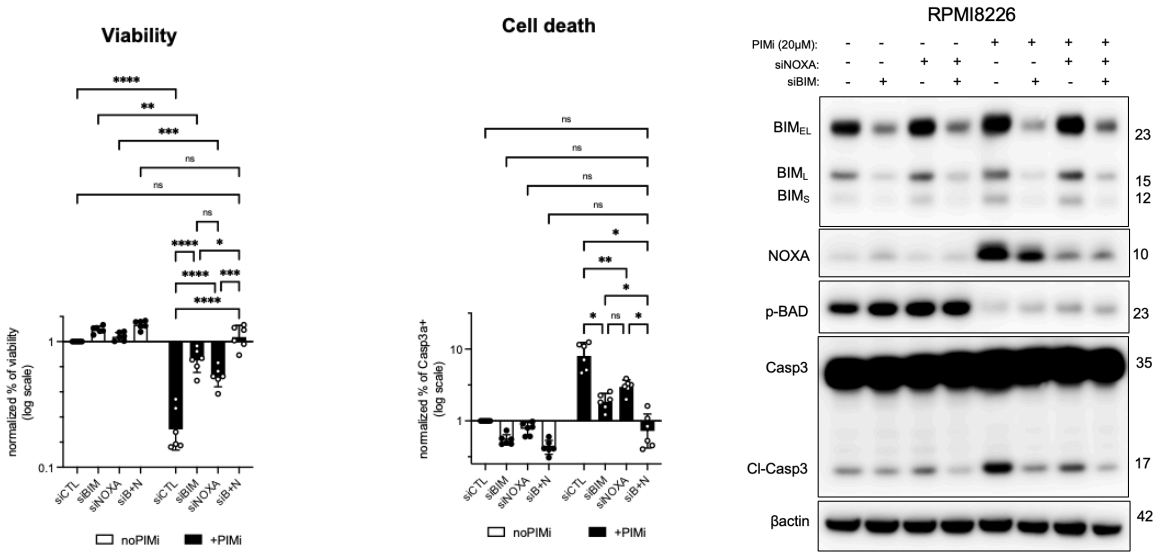


We have modified supplementary figures and the text of the manuscript (Lines 64-74) accordingly.

2. **MAJOR POINT**. Unless I have missed it, the only data that directly shows the impact of combined PIMi and NOXA loss is Figure 2F, which shows a modest impact for primary ASCs. What is the survival impact for combined PIMi and NOXA knockdown in the MMCL?

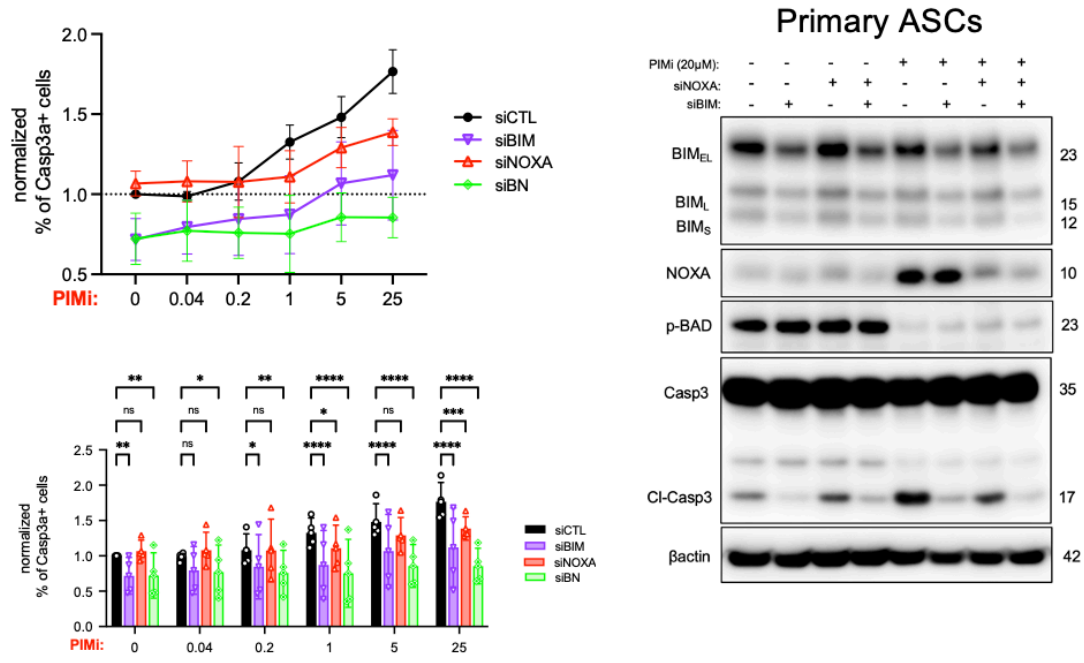
Response:

We agree with the reviewer that the effect of siNOXA in primary plasmablasts shows only a moderate reduction in active caspase 3, as indicated by flow cytometry (Fig. 2f). As suggested, we tested the impact of siNOXA on the RPMI8226 cell line, alongside the siBIM experiment mentioned in the following question. We assessed both cell viability and cell death (measured by the percentage of Caspase 3-active cells) after 48 hours of AZD1208 treatment. The results, presented below, demonstrate a significant decrease in cell death and a marked recovery in cell viability with siNOXA treatment compared to the control condition (siCTL) following PIM inhibitor treatment. The immunoblot confirms that our siRNA strategy was effective, resulting in complete knockdown of both BIM and NOXA expression. Additionally, our data show that the induction of NOXA under PIMi is abolished by siNOXA, leading to a reduction in Caspase 3 cleavage compared to the control. Finally, the results highlight the efficacy of siRNAs, particularly when used in combination, in limiting Caspase 3 cleavage induced by PIMi.



These results have been included in the revised version of our manuscript in Fig; 2g. In this manuscript, we decided to present only the statistical results most relevant to our study. The modifications in the manuscript are in lines 141-145.

Given the particular interest in studying the distinct contributions of NOXA and BIM in MMCLs, we extended this investigation to in vitro generated primary plasmablast. Our findings revealed that siBIM was significantly effective in the basal state to lower Caspase 3a-positive cells, i.e., without PIMi treatment, while siNOXA had no effect. This outcome aligns with the high expression of BIM in plasmablasts and the negligible presence of NOXA under control conditions.
Upon PIMi treatment, siBIM showed significantly greater efficacy than siNOXA, though the effect was still less than that achieved by combining both siRNAs. These results indicate that, while BIM plays a prominent role in apoptosis in our primary cells, NOXA also specifically contributes to apoptosis induced by PIMi.



These results have been included in the revised version of our manuscript in Fig. 2h. The modifications in the manuscript are in lines 146-153 and 657-659.

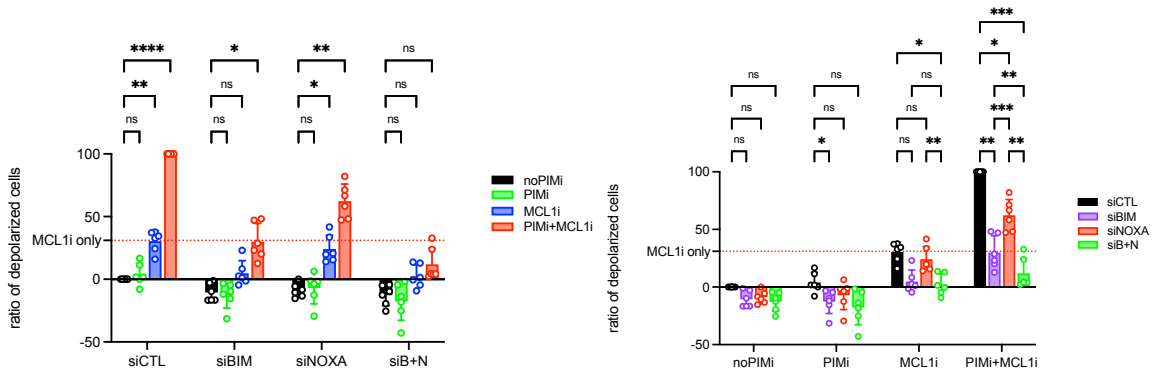
3. A related point is the role for BIM in promoting apoptosis. BIM's function gets less attention as it is not transcriptionally induced like NOXA, and has lower affinity for MCL1, but it is still likely a key component of the cell death after PIM/MCL1 inhibition. Parallel PIM/BIM knockdown would answer this question.

Response:

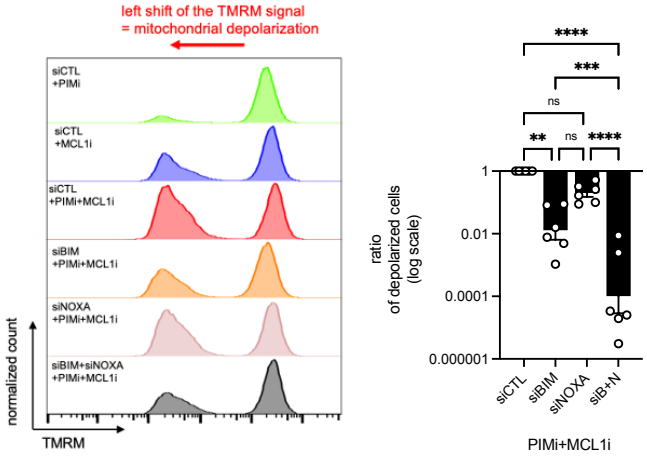
We thank the reviewer for the insightful comment regarding BIM and its role in inducing apoptosis. As mentioned in our response to the previous question, we investigated this issue using an siBIM in the RPMI8226 cell line. After 48 hours of AZD1208 treatment, we observed a significant reduction of cell death induced by the PIM inhibitor compared to the control (siCTL). Note that the effect of siBIM alone was greater than that of siNOXA alone in countering PIMi induced apoptosis. Interestingly, the combined effect of siNOXA and siBIM was significantly more effective than that of either siRNA alone. These results are highly relevant and indeed confirm that the effect of PIMi in inducing apoptosis is mediated by BIM and NOXA, whose effects complement each other.

Given these findings, we further investigated the impact of BIM and/or NOXA depletion on the synergy between PIM and MCL1 inhibitors in RPMI8226 cells. The synergy is compromised by the depletion of either siNOXA or siBIM, with BIM depletion having a more pronounced effect than NOXA. This is likely because BIM influences MCL1i activity on its own, whereas NOXA has little impact on MCL1i alone—likely due to its low expression and lack of binding to MCL1 without PIMi treatment. Finally, the results demonstrate that depleting both molecules has a significantly greater effect on reducing the synergy of the drug combination compared to depleting a single molecule. This underscores the critical role of these two factors in the synergistic action of these drugs (results presented below).

Panel A



Panel B



These results have been included in the revised version of our manuscript in lines 379-380 and Fig. 5g (Panel A, left) and Supplementary Fig. 5m (Panel A, right) and 5n (Panel B).

4. How is NOXA induced? The data in Figure 3H bottom shows that the block after ISRIB/PIMi is at the transcriptional level, and other data suggest that p53 is not involved. Do the authors at least have a model to explain this?

Response:

NOXA production following kinase inhibition, specifically of PIM2 and not PIM1 (as shown in blot lanes 3 and 4 in Fig. 3j), is driven by the production of the transcription factor CHOP, which is induced by the integrated stress response (ISR). This, in turn, leads to the transcription and production of NOXA (see qPCR panels for *DDIT3*/CHOP and *PMAIP1*/NOXA at the bottom of Fig. 3j). The involvement of p53 is entirely excluded, as confirmed by our results using the p53-depleted XG7 cell line, which were provided in response to Reviewer #1 (see above, response to major comment “G”).

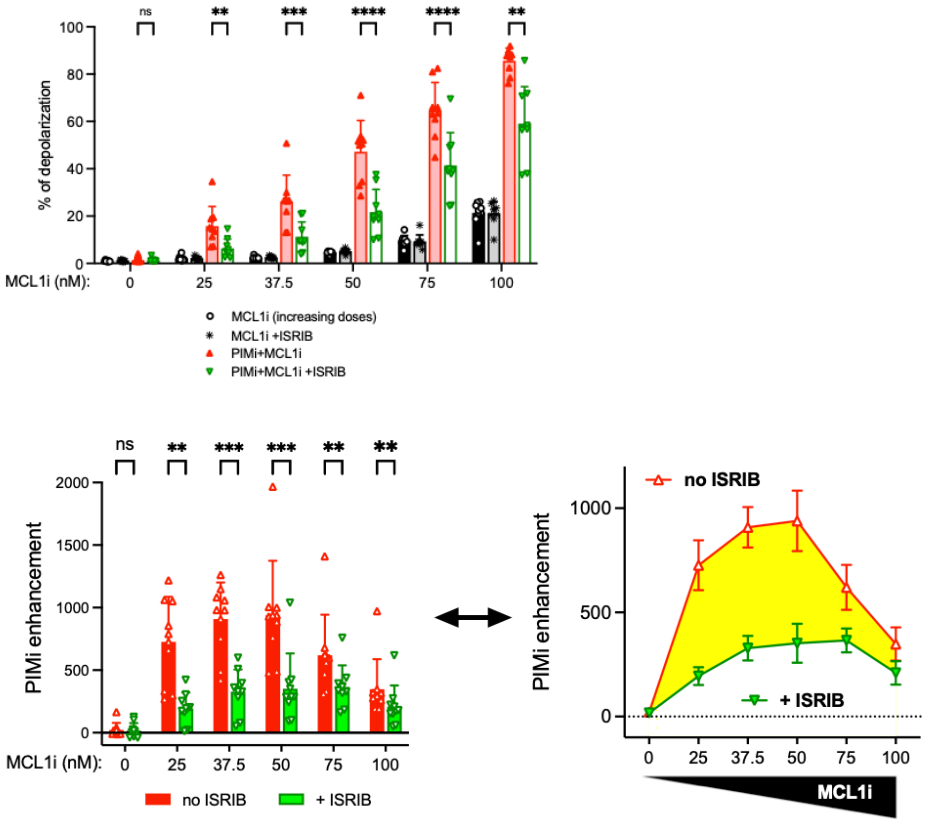
We have revised the manuscript to clarify the description in the third paragraph of the “result section” and added results in Fig. 3.

5. Figure 5F. I am surprised that the value comparing PIMi/MCL1i +/- ISRIB are statistically significant, given the low numbers of experiments and the high variation. Please check these calculations.

Response:

As recommended by the reviewer, we have reconsidered the analyses of this experiment. A series of 7 new independent experiments were performed in order to obtain clear and significant results (data presented below).

The Fig. 5h (mitochondrial depolarization) and Fig. 5i (PIMi enhancement score) have therefore been completely revised in the resubmitted manuscript.



Minor comments

6. It appears that the western blots in Figure 1A-B and again in panel E, duplicate the b-actin loading control. This is OK but should be mentioned in the legend.

Response:

Thank you for this advice.

In fact, we used the same protein extracts to detect the different proteins for the two Western blots in panel 1B and the two Western blots in panel 1E. Therefore, the β-actin control was performed only once for each experiment but is shown for each western blot.

In the revised version of the manuscript, we have changed the legend of Fig. 1 for panels 1b and 1f accordingly. In addition, in the "method section" we've specified this point (lines 921-924).

7. Line 243. Should be suppl Fig 4C.

Response:

Yes, that's right; thank you for your vigilance. As pointed out in one of the questions from reviewer #1, this error on our part contributed to making our result more difficult to understand.

In the revised version of our manuscript, we have made the correction. In addition, as mentioned above, we have slightly modified the text to improve the understanding of this result (lines 313-317).

8. Line 257. The conclusion that ISRIB suppresses NOXA recruited to MCL1, appears an overinterpretation as ISRIB blocks Noxa transcription in the first place.
Response:
In fact, ISRIB blocks, at the transcriptional level, the induction of NOXA upon PIMi treatment. As a result, NOXA protein is not produced and NOXA will obviously not be recruited to MCL1 in this condition. The aim of our IP Fig. 4h was to confirm and illustrate the absence of NOXA on MCL1 in the ISRIB condition, despite PIMi.
We have revised the sentence in the manuscript to read: " As expected, the addition of ISRIB, which prevents NOXA induction under PIMi, decreases the amount of NOXA on MCL1 cells despite PIMi treatment" (lines 326-328). We hope this new interpretation is acceptable.

9. The statistical significance p values (*, ** etc) are not defined anywhere in the manuscript.
Response:
We apologize for this oversight; in the revised version of our manuscript, we have added the definition of p-values at the end of each legend.

Reviewer #3 - PIM2 (Remarks to the Author):

What are the noteworthy results?

The manuscript entitled “Uncovering the Role of PIM2 in Modulating MCL1 Dependency and Activation of ISR-Mediated NOXA Expression” details an extensive body of work undertaken by the authors, in normal and malignant plasma cells, that examines the intricacies of the integrated stress response (ISR) axis, and in particular the pivotal role of PIM2 kinase and its interaction with the BCL2 family of proteins, highlighting crucial mechanisms that regulate cell survival and tumor progression.

The manuscript delivers several noteworthy results:
1. Inhibition of PIM2 kinase suppresses pBAD levels and activates the Integrated Stress Response (ISR) pathway inducing NOXA and Caspase 3 in both antibody-secreting cells (ASCs) and multiple myeloma (MM) cell lines.

2. NOXA's role in Caspase 3 activation remains significant regardless of p53 expression and is distinct from the decline in pBAD levels, directly attributed to PIM kinase inhibition.

3. ISRIB inhibition of the ISR in combination with AZD1208 or antisense morpholino PIM inhibition in ASCs, suppresses ATF4/CHOP expression, prevents NOXA induction and Caspase 3 activation independently of pBAD inhibition (Figure 3G) and improves cell viability (Figure 3H and Supplemental Figure 3C).

4. PIM inhibition triggers a cascade of events (Figure 4I) which results in the loading of pro-apoptotic BH3-only proteins onto MCL1. Increased BAD binding to BCL-XL frees BIM and BAK, relocating them to the mitochondrial membrane where they engage with MCL1 along with NOXA. MCL1 thus becomes saturated with proapoptotic BH3-only proteins which accentuates apoptotic priming.

5. PIM inhibition facilitates more effective mitochondrial depolarization induced by MCL1 inhibitor AZD5991, particularly in AZD5991-sensitive MM cell lines. Specifically, PIM2 depletion, but not PIM1 via morpholinos amplifies the response to AZD5991-induced mitochondrial depolarization. PIM inhibition is required to free BAK from MCL1 and prevents BAK and BIM binding to BCL-XL thus activating BAX, triggering MOMP, mitochondrial depolarization, and cytochrome c release, even at lower doses of MCL1 inhibitor in susceptible MM cell lines. PIM inhibition alleviates resistance to MCL1 inhibition.

6. In vivo, enhanced myeloma xenograft regression via the synergistic effect of combining PIM and MCL1 inhibitors, demonstrates improved outcomes compared to single-agent treatments. These data provide a rational for co-targeting treatment strategies in MM.

Will the work be of significance to the field and related fields?
How does it compare to the established literature?

Yes, this large body of work, that examines in-depth the role of PIM2 inhibition in apoptotic priming, adds significant value to the field of PIM kinases and targeted

therapies for the treatment of multiple myeloma (MM) and other cancer types. It was previously highlighted in the literature that PIM2 is highly expressed in MM, mediating proliferation and promoting cell survival. It also has a key role in the bone destruction typically seen in MM and therefore constitutes a promising therapeutic target.

However, PIM2's function in MM is multifaceted and a thorough understanding of all its roles is required to enhance drug development and guide optimal treatment strategies that can effectively cause cell death without activation of alternate resistance pathways. It will be important to elucidate any conferred functional overlap with PIM1 and PIM3 which has not previously been investigated in detail.
The established literature does highlight all 3 PIM kinases are expressed in MM cells and the PIMs act as pro-survival factors to phosphorylate Bcl-2-associated agonist of cell death (BAD) and pre-vent apoptosis. PIM kinases are upregulated in the CD138 + fraction of patient bone marrow. High PIM2/3 protein expression, across the majority of a panel of MM cell lines, was demonstrated by Ramachandran, J., et al. PMID: 27564460 (Figure 1b). Single transient knockdowns of PIM1/2/3 in MM1.S cells via siRNA transfection functionally separated the three isoforms. PIM2 knockdown alone had significant effects on cell survival at 24 and 48 h after transfection and was the only isoform implicated in the activation of the apoptotic pathway (Figure 2) whereas PIM 1 and 3 did not result in apoptosis.

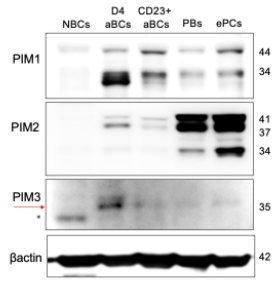
Response:
Thanks to the reviewer for this suggestion to explore in the context of our PIM3 study to rule out any redundancy or compensation by this kinase in the context of ASCs.
We have performed a large number of new experiments, which are described below.

Since this question of the role of PIM3 in myeloma remains open and was not addressed in our manuscript submitted to the reviewers, we wanted to bring some new information to the table.
First, in the context of Figure 1 and the characterization of the 5 B-cell populations obtained in our in vitro differentiation model of primary human B cells, we detected the expression of PIM1, PIM2 and PIM3 proteins for each population (this result has been included in the revised version of our manuscript in Suppl Fig. 1a, b and is presented below, panel A). As can be seen, PIM3 expression is low and difficult to detect.
An increase in PIM3 expression was observed in activated B-cells at d4 (D4-aBCs), probably related to the presence of memory B cells in this population; other data (genomic scape, Jérôme Moreaux's data), not presented here, suggest the presence of PIM3 in memory B cells.

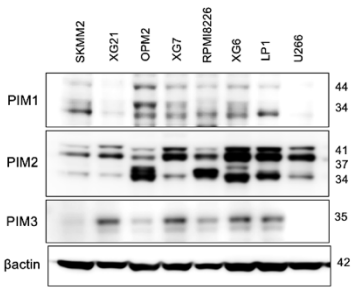
In the revised manuscript, we included the results of immunoblot detection of PIM3 in B cells subsets in our in vitro model and comments were added at line 49 (the blot was added as Supplementary Fig. 1a, b and is shown above, panel A).

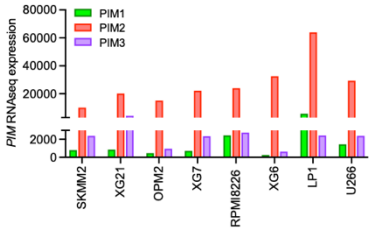
A similar analysis was conducted for the eight multiple myeloma cell lines (MMCLs) used in our study, with the results included in the Supplementary Results as Suppl Fig. 1h of the revised manuscript (Panel B shown below). As observed, PIM3 expression varies among the MMCLs. RNA-seq data from Jérôme Moreaux's team indicate that PIM3 mRNA expression is comparable to that of PIM1 but significantly lower than that of PIM2 (right graph on panel B below).

Panel A



Panel B

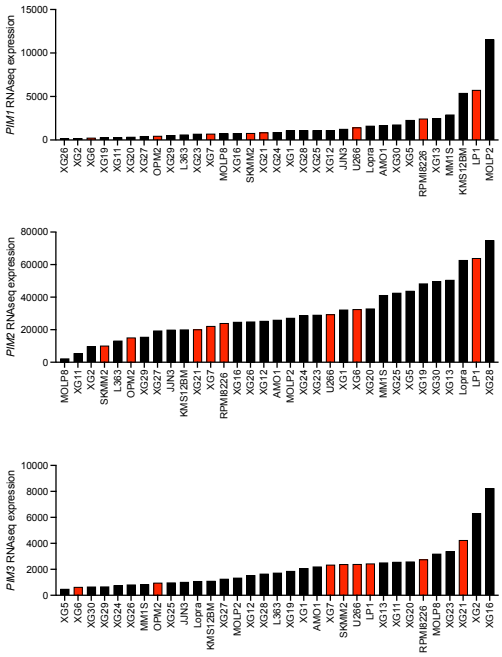




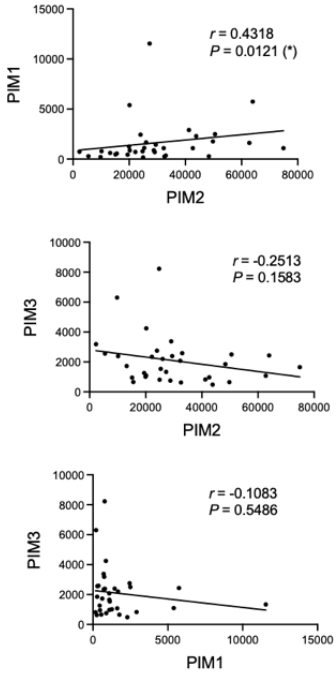
We also checked the expression of the three PIM kinases across the 33 multiple myeloma cell lines (MMCLs) using RNAseq data provided by J. Moreaux's group (CNRS, Montpellier, France). The RNAseq data revealed a significant difference in expression levels, with *PIM2* gene showing much higher signal intensity (number of reads) compared to *PIM1* and *PIM3*. Using these data, we also investigated whether there were any correlations in PIM gene expressions when they were analyzed 2 by 2. The only significantly positive correlation detected concerned PIM1 and PIM2.

Results are presented below in panel C & D. The panel C was included in the revised version of the manuscript in Supplementary Fig. 1g.

Panel C



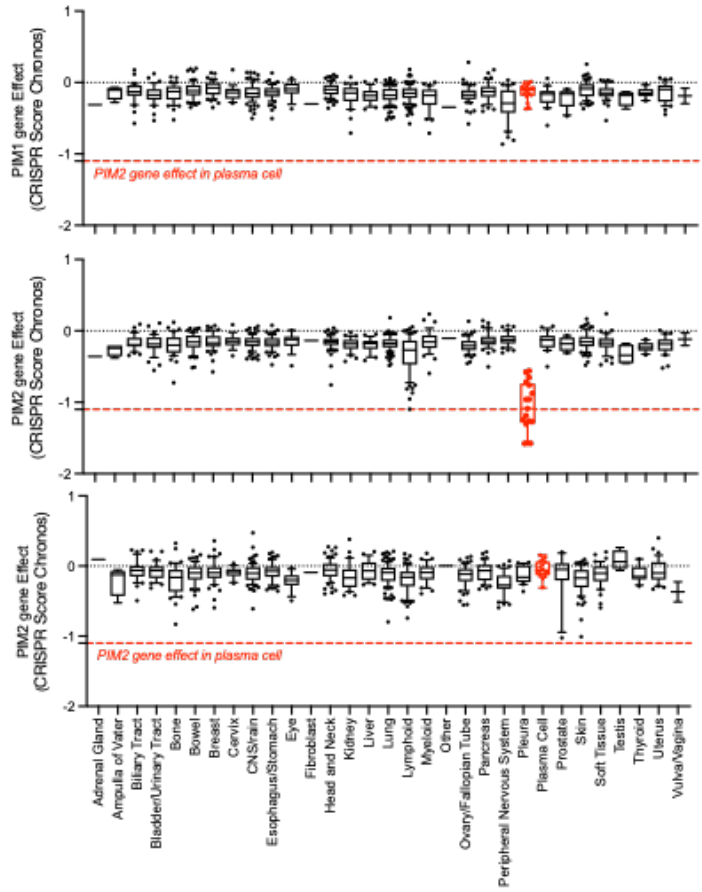
Panel D



Finally, we again interrogated the DepMap Portal project in silico for the dependency of the approximately 800 tumor cell lines analyzed after CRISPR-Cas9 gene deletion (methodology previously described in Haas et al. 2022, PMID: 35108359). These results clearly show that only *PIM2* gene expression is essential for cell survival of MMCLs, which is not the case for *PIM1* and *PIM3*.

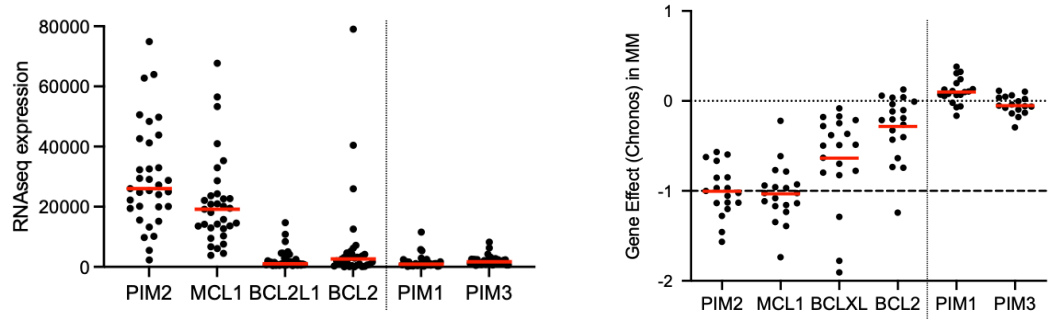
This result is shown in panel E below but is not included in the revised version of the manuscript.

Panel E



The initial figures in the manuscript showing only PIM1 & PIM2 (RNAseq and Gene Effect data) have been supplemented with PIM3 and are shown in Fig. 1d in the revised version of the manuscript (below the updated Fig. 1d, panel F).

Panel F



The authors state that "…PIM2 inhibition activates the ISR-related ATF4/CHOP axis, leading to NOXA protein expression concurrent with pBAD inhibition. This represents the first evidence of PIM2's involvement in the integrated stress response (ISR) pathway, expanding our understanding of its pivotal role in regulating cell survival in PCs". However, previously in prostate cell lines, the Kraft group demonstrated that PIM

inhibition transcriptionally increased levels of the BH3 protein Noxa by activating the unfolded protein response (UPR), lead to eIF-2α phosphorylation and increased expression of CHOP. Increased levels of Noxa also inactivated the remaining levels of Mcl-1 protein activity PMID: 22080570. This previous data should be highlighted here in this manuscript by the authors.
Response:
We agree that the work of A. Kraft's group in prostate cancer is significant and has clearly demonstrated the increase in NOXA following PIM kinase inhibition. In the initial version of the manuscript, we referenced this paper (as reference number 5). In the revised version of our manuscript, we have recalled this reference within the discussion section (Lines 636-638).
Regarding the decrease in MCL1 protein, as mentioned in our response to reviewer #1, our work in ASCs suggests that this reduction is likely not due to proteasome activity following NOXA binding. Instead, it appears to be mediated by degradation through caspase activity, a process previously described by Herrant et al. (2004, PMID: 15378010; ref #16 in our paper).

Does the work support the conclusions and claims, or is additional evidence needed?
For the most part the work supports the conclusions and claims however authors should address the following:

The pan-PIM inhibitor AZD1208 is used throughout and authors state in line 56 "…in parallel with a PIM inhibitor targeting PIM2…" and line 105 "To explore this relationship, we examined the impact of PIM2 inhibition using the PIM inhibitor AZD1208" – all 3 PIM kinases will be inhibited by AZD1208 so PIM2 alone not inhibited by AZD1208.
Response:
I understand the reviewer's concern regarding our use of the term "PIM2 inhibition" throughout the manuscript, given that AZD1208 is a pan-PIM kinase inhibitor. We adopted this simplification because our work clearly demonstrates that in ASCs, it is specifically PIM2, not PIM1 or PIM3, that plays a key role in controlling the ISR and NOXA production. Our findings, supported by experiments using morpholinos specifically targeting each PIM kinase (as presented below), reinforce this conclusion.

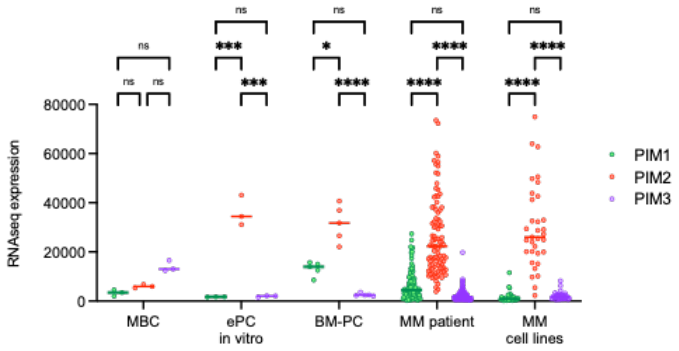
We have revised the manuscript in several places including lines 119-120 (initial manuscript, line 105) to clarify this point, now referring to AZD1208 as a pan-PIM inhibitor rather than exclusively as a PIM2 inhibitor.

The SSO experiments target both PIM1 and PIM2 but not PIM3 which has been shown previously to be highly expressed across the MM cell lines used. Although it has been shown previously that PIM2 might by the only PIM kinase regulating apoptosis the authors should address the fact that all 3 PIM kinases are expressed in MM cells and that there is evidence that there is compensatory potential between PIM kinases. The data would benefit from the addition of PIM1 and PIM3 protein expression data in the panel of MM cell lines (Figure 1E) or at least to highlight previous published data in PMID: 27564460 (Figure 1B).
Response:
In response to the previous reviewer's question, we presented above a series of new results regarding the expression of the three PIM kinases in MMCLs.

To further support our answer, we have analyzed RNAseq data from our laboratory and J. Moreaux's group. These results, shown below in panel A, depict RNAseq read counts across various cell populations: MBCs (primary memory B cells), ePC in vitro (in vitro-produced primary plasma cells), BM-PC (primary plasma cells from healthy volunteers), MM patients (a cohort from the hospital in Montpellier, France, as described in our previous paper, Haas et al. 2022, PMID: 35108359), and the 33 previously described MMCLs.
These data confirm the predominant expression of PIM2 in plasma cells, significant PIM1 expression in mature bone marrow plasma cells or certain myeloma patients, and generally low expression of PIM3.

Although these results are presented below, they are not fully included in the revised manuscript. Only the data on MM patients and MM cell lines have been added in the revised manuscript in lines 50-54 (graph is now in Supplementary Fig. 1c).

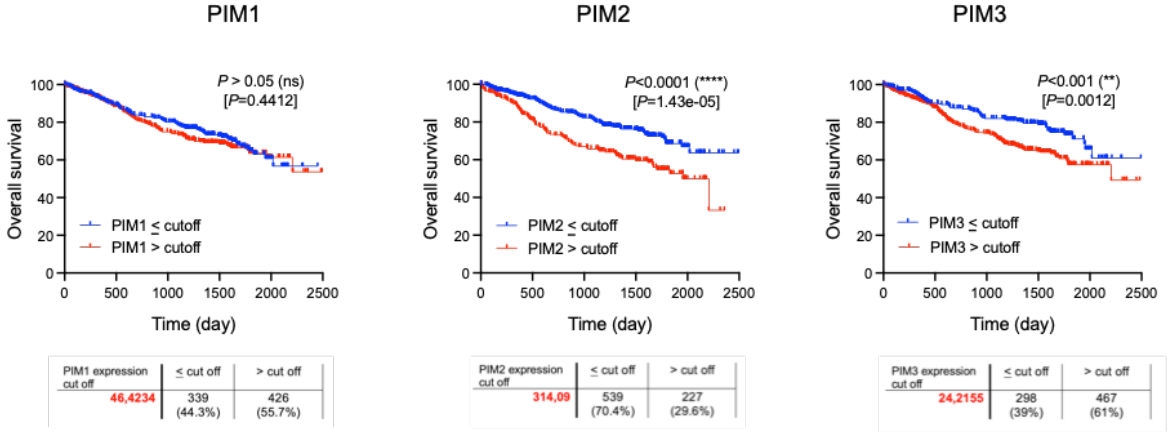
Panel A



To further support our findings on PIM kinases in multiple myeloma, we present in silico data on overall patient survival from the CoMMpass cohort (methodology described previously in Haas et al. 2022, PMID: 35108359). A cutoff for each PIM kinase was determined using the MaxStat method, as described in that publication. These results, shown below in panel B, clearly highlight the predominant role of PIM2 expression in myeloma, with PIM3 also showing a significant difference in OS, unlike PIM1.

While these intriguing data are presented below, they have not been included in the revised manuscript, as they fall outside the scope of our study.

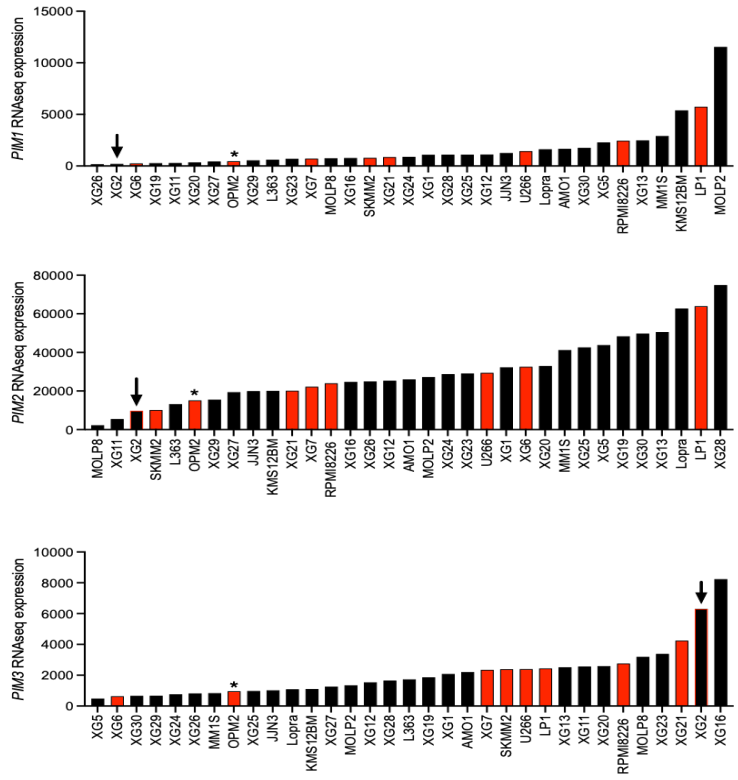
Panel B



Also, authors should comment on the OPM2 cell line which has a weak PIM2 expression (low MCL1 & low NOXA) and very high pBAD levels, compared to the other MM cell lines (Fig 1E/F). Interestingly, PMID 27564460 (Figure 1b) shows OPM2 cells have low PIM1/PIM2 expression, high PIM3 expression and low pBAD expression compared to those with higher levels of PIM2.
Authors show pBAD is decreased with PIMi in OPM2 cells (Figure 2b) and the IC50 dose for AZD1208 is 11.2uM while U266 cells have high PIM2 and MCL1 protein expression & IC50 dose for AZD1208 is 12.2uM. As mentioned above PMID: 27564460 (Figure 1b) shows OPM2 cells express high PIM3 expression; the authors should comment on whether PIM3 might also activate BAD in this cell line. It would be interesting to examine the effect of SSO PIM1/PIM3 & SSO PIM2 in OPM2 cells and determine whether the response to PIMi, siNOXA are similar to that seen in U266 and RPM8226 cells.

Response:
As shown below, we complemented the manuscript with investigations on PIM3 expression in MMCLs. Firstly, as show above and added in Supplementary Fig. 1h of the manuscript (immunoblot on MMCLs), the expression of PIM3 in OPM2 MMCL is very low. These findings are consistent with those obtained by J. Moreaux's group (CNRS, Montpellier, France) in their RNA-seq analysis of 33 MMCLs (panel A). Overall, our results indicate that OPM2 expresses the three PIM kinases at relatively low levels as indicated with stars in each graph on the panel A.

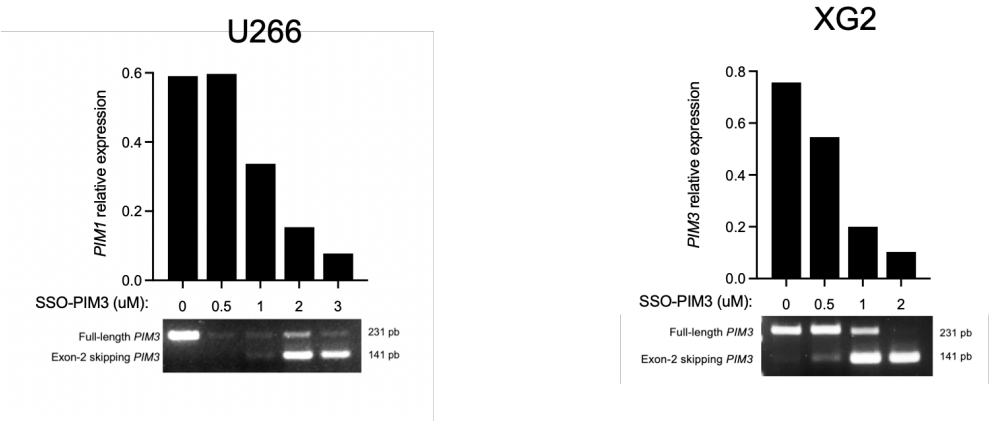
Panel A



Following the results and responses provided in relation to the OPM2 cell line, we now address the reviewer's question regarding whether PIM3 is capable of phosphorylating

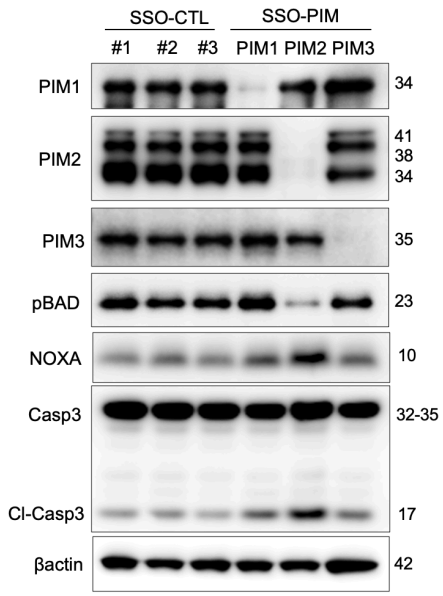
BAD. In Fig. 2c of the manuscript, we demonstrated that PIM1, when targeted by a morpholino, does not have this capability.
With the assistance of Laurent Delpy (CNRS, Limoges, France), who has been added as a co-author in the revised manuscript, we designed a morpholino specifically targeting PIM3. The functional validation of this morpholino was conducted on U266 and XG2 cells, where we observed clear exon skipping, producing a 141bp band that led to a significant decrease in PIM3 mRNA expression (panel B, below).

Panel B (updated in Suppl Fig. 2b, only with U266 data, in the revised version of the manuscript)



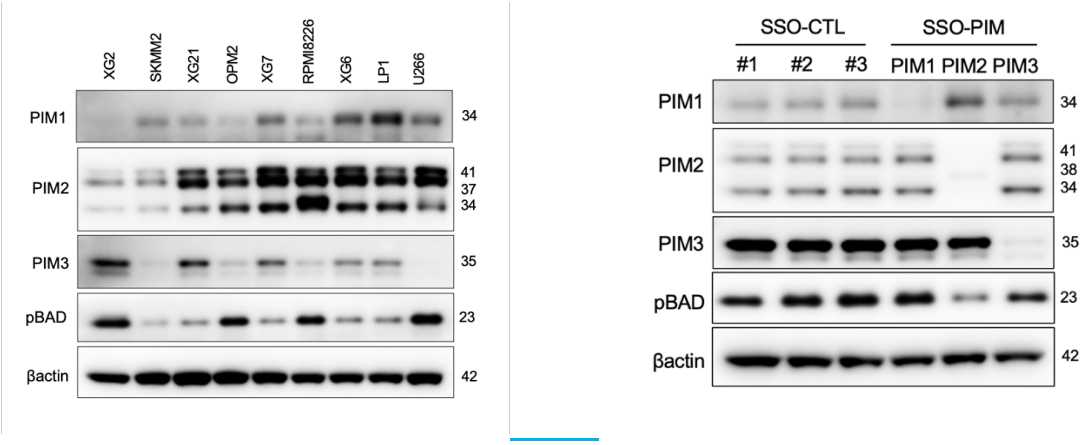
We then compared the effects of the three morpholinos used in our study, each specifically targeting PIM1, PIM2, or PIM3. For this experiment, we selected the LP1 cell line, as it strongly expresses all three kinases PIM and pBAD. Our results demonstrate the clear efficacy of these morpholinos in specifically inhibiting their respective PIM targets. Notably, only PIM2 was found to be indisputably involved in BAD phosphorylation (panel C, below).
We further analyzed these protein extracts obtained from LP1 cells. The ISR pathway was most strongly activated under the PIM2 morpholino condition, leading to increased NOXA production and enhanced caspase 3 cleavage (panel, below).

Panel C (updated in Fig. 2c)



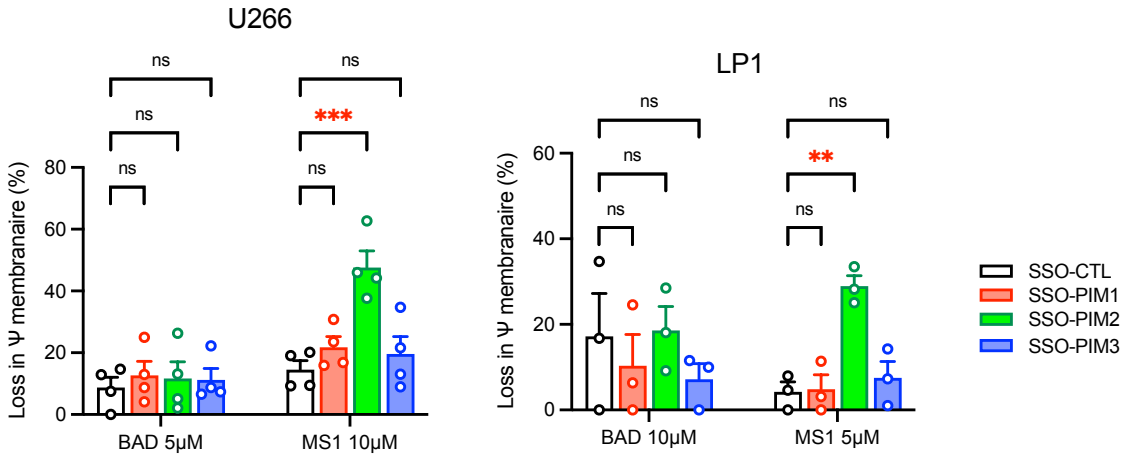
Finally, although OPM2 strongly expresses pBAD, the expression of PIM3 is very low and we therefore considered that it was not very relevant to use this line to investigate the role of PIM3 on pBAD.
Considering the RNA data from Jerome Moreaux' team, we identified the XG2 cell line, expressing PIM1 and PIM2 very weakly, but PIM3 strongly (as indicated with arrows on the panel A above). Immunoblots analysis on the 8 MMCLs to which we added the XG2 cell line, confirmed these data (below left, panel E). We therefore thought that it might be interesting to use this line for exploration with SSO. We therefore optimized the efficacy of the 3 SSOs on this new cell line (see above panel B right) and assessed the effect on pBAD (below right, Panel F): we then observed a reduction in pBAD only in the SSO-PIM2 condition. Thus, despite the high expression of PIM3 in this line, and the low expression of PIM2, it is always and exclusively PIM2 that plays a role in pBAD (Panel F).

Panel E & F (not included in the revised version of the paper)



Finally, to test the effect of PIM3 on anti-apoptotic factor dependencies, we performed the BH3 profiling assay using the MS1 (for MCL1) and BAD (for BCLXL/BCL2) peptides on myeloma cells (LP1 and U266 MMCLs) treated with SSO-PIM3. Our results confirm that PIM2 specifically increases MCL1 dependency and show that PIM3 knockdown has no effect on either MCL1 or BCLXL/BCL2, as PIM1.

Panel G



Extensive data and investigations on PIM3 and its effects have been included in the revised version of the manuscript in Fig. 2 (Fig. 2c and Suppl Fig. 2b) and Line 125 of the manuscript. The panel G is now the Fig.4b.
The data concerning XG2 have not been included since this cell line was not used for the other explorations. This cell line was just used as a tool to argue a relevant question of the reviewer.

Authors should clarify differences in PIM2 isoform expression in RPMI8226 cells in Figure 1e (all 3 expressed) and Figure 2c (CTL1 and CTL2 mainly 34kD expressed)?
Response:
We agree that the initial version of Figure 2C on RPMI8226 cells showed only the 34 kDa band of the PIM2 protein. We included these results in our paper to illustrate the increase in NOXA and the corresponding decrease in pBAD following specific PIM inhibition using morpholinos, a result that was positive for RPMI8226 cells.

In the revised version of our paper, we propose to demonstrate these effects using the LP1 cell line, which displays all three bands of the PIM2 protein (Fig. 2c).

Can authors comment on Figure 2A which has low levels of PIM1 expression with SSO PIM1 and complete inhibition of PIM2 with SSO PIM2? Interestingly, NOXA expression does increase slightly with SSO PIM1 (Figure 2A, 3B & 3H) albeit that SSO PIM2 has a larger increase in NOXA expression. Similarly Figure 2C cell Line RPM8226 with SSO PIM1 has a slight increase in NOXA & cleaved caspase expression although pBAD does not decrease.
Response:
We agree with the reviewer's comments regarding the imperfect efficacy of PIM1 inhibition under SSO and the weak induction of NOXA following specific PIM1 inhibition. Although morpholinos are generally very effective—requiring no electroporation to enter cells—we have observed in some experiments, and depending on the cell type studied, that their efficacy in inhibiting the target PIM can vary. Consequently, in primary ASCs we often need to adjust morpholino doses according to each healthy donor. In this study, we used a standardized dose across all experiments for primary cells as well as MMCLs analyzed; unfortunately, sometimes it is probably a bit too low (probably as here) or too high (risk of inducing toxicity).
While we found that only PIM2 effectively induces BAD phosphorylation in primary ASCs, it appears that PIM1 may have a modest effect on the ISR pathway and inhibition of NOXA production following cellular stress. As mentioned, PIM1 is expressed in both normal and tumor mature plasma cells, and depending on its localization and expression level, we cannot entirely rule out some contribution of PIM1 kinase to the ISR. However, as the reviewer correctly pointed out, this effect is minimal compared to the significant impact of PIM2 inhibition on NOXA production.

Are there any flaws in the data analysis, interpretation and conclusions?
I have not identified any significant flaws in the data analysis, interpretation or conclusions in this manuscript

Do these prohibit publication or require revision?
Minor revisions as detailed above and below.

Is the methodology sound? Does the work meet the expected standards in your field?
Yes the methodology is sound and the work is of an exceptional high standard in the field.

Is there enough detail provided in the methods for the work to be reproduced?
Yes there is enough detail provided in the methods for them to be reproduced.

Further edits and Typos:
Figure 2c & 2e title of graph should be “RPMI8226” not “RPM8226”
Response:
Thanks, we’ve corrected the title accordingly.

Figure 3a “Noxa” not “IOXA” and include PIMi (24hrs) on graph on right
Response:
Thanks, we’ve corrected the typo accordingly.

Figure 4I “Hight” to “High”
Response:
Thanks, we’ve corrected the typo accordingly.

Figure 5C &5D Image for BAK in RPMI cells &XG7 cells has been cropped too close to 23kD band removing part of the upper band?
Response:
The images in Fig. 5 have been cropped. However, the truncated band does not correspond to a BAK band, but to the light chains of 25KD immunoglobulins. For this series of IP experiments, we have used Protein B beads that reveal these light chains.

The blots are provided as uncropped blots in Source Data, following recommendations of the journal, so that the 25KD band can be easily identified.

Figure 3E is there a pBAD image for U266 cell line?
Response:
For the initial Figure 3E, which has now become Fig. 3g in the revised version of our manuscript, we performed a new immunoblotting with the remaining proteins extracted from U266 cells and stored in the laboratory.
The pBAD result was thus added to the other proteins detected in this experiment.

Figure 3F is the casp3 image for RPMI8226 cell line?
Response:
In the original Figure 3F, which became Figure 3h in the revised version of our manuscript, we did not show total caspase-3 for RPMI8226 cells because the amount of protein was so large that we had difficulty correctly visualizing the cleaved caspase-3 signal. In this figure, we have initially shown total caspase 3 for primary ASCs and the U266 cell line.
To maintain homogeneity between the different western blots in Figure 3f, g and h, we removed the total caspase 3 band. The cleaved caspase-3 result is now clearly shown and allows for a correct assessment of apoptosis activation.

Reviewer #1 (Remarks to the Author):

This reviewer appreciates the work and effort the authors have put into improving their manuscript and responding to this reviewer´s concerns. The authors have satisfactorily replied to most of this reviewer´s concerns. However, some replies, naturally, are dissatisfying.
Nevertheless, this reviewer feels that the improvements and replies now justify the publication of the manuscript.

Some minor comments are listed below:
a) Labelling in figures throughout seems to be partially imported as pixel-based image. This is unfortunate since sub-figures are rather small. When zooming in, text becomes pixelated.
We've taken the reviewers' comments into consideration and will re-import the sub-figures into our files so that they don't appear pixelated when zoomed in.

b) regarding the original minor comment F) of this reviewer: pBAD is understood to be phosphorylated-BAD. When showing anti-pBAD western blots, usually anti-BAD western blots are expected. Reduced band intensity for pBAD should result from suppressed/blocked phosphorylation (or enhanced dephosphorylation). In a narrower sense, the molecule pBAD itself can not be "silenced" - "silencing" is from this reviewer´s experiencd primarily used in the context of gene expression.
The reviewer is right. pBAD is not silenced in the proper definition of the term. pBAD corresponds to the phosphorylated form of the BAD protein, which is suppressed in the presence of the PIM inhibitor, without affecting BAD expression. We have corrected the sentence in the text.

c) Regarding to original minor comment G). This reviewer has clearly understood what the meaning of "notable expression pattern" is. I apologize for not clearly stating the concern. The intended question was: what exactly is notable about the expression pattern, please specifiy in which way the expression pattern distinguishes from the "not notable" expression pattern.
This sentence concerns the kinetics of NOXA expression during the differentiation of LyBs into PCs.
The expression profile of NOXA is indeed, in our view, intringent, since it shows a peak of expression in activated LyBs that contrasts with a very low expression in other populations.
For our explorations, we are mainly interested in antibody-secreting cells (ASCs), i.e. PBs and ePCs. In these two populations, NOXA expression is very low or even extinct. This correlates perfectly with our further investigations, which showed that the ISR pathway that induces NOXA is indeed blocked in ASCs.

d) regarding original comment mjor H and minor K): this reviewer very much appreciates the immunofluorescence microscopy of cytochrome c release (also by FACS) and IF of activated (i.e. active) BAX! Thank you very much for the effort! Just for clarification: the subclone antibody 6A7 is specific for the N-terminur of BAX and therefore detects BAX exclusively in it´s active conformation (or denatured).
Thanks to the reviewer for his comments and clarification.