nature portfolio

Peer Review File

Chemotherapy resistance in acute myeloid leukemia is mediated by A20 suppression of spontaneous necroptosis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the Starczynowski laboratory identifies A20 as an important downstream effector of chemoresistance in AML. The authors present data that proposes A20 induced NF-kB expression leading to activation of a chemo-resistance gene network highlighted by A20's ability to impair necroptotic cell death. Their hypothesis was generated by gene expression analysis of publicly available AML data sets with supportive functional studies in both human AML cell lines, primary AML patient samples and A20 knock-in and knock-down mouse models. They elevate their work by conducting thorough mechanistic studies by combining their A20 knock-out model with chemical and genetic inhibition of various hypothesized effectors of A20 pro-leukemia function, coming to the conclusion that A20 promotes leukemia growth and doxorubicin resistance by suppressing RIPK3-mediated necroptosis through its E3-ligase function rather than through mechanisms involving its negative-feedback on NF-kB.

While it is widely known that A20 signals through the NF-kB, this ambitious manuscript sought a deeper understanding of cell-intrinsic chemo-resistance in AML induction therapy that goes beyond previous efforts which have stopped at descriptions of correlations seen between chemo-resistance and NF-kB, such as with BCL2 family protein upregulation. The problem of chemo-resistance and induction failure is a highly relevant unmet need in AML treatment, and new insights into this problem have a chance for high clinical impact. Their work identifies a new biomarker correlated with chemo-resistance (A20 high expression) and has clear and easy to follow implications for the design of new therapies targeting this mechanism of cell death resistance. Only at the highest expression levels of A20 (90th percentile) is there a strong correlation with clinical outcomes, which limits relevance to a small population of refractory patients, but the insights into the cellular biology could lead to effective drug development outside of this small population. In general, this paper is professionally written, and shares novel data about a common clinical conundrum, using appropriate methods and controls.

Comments to address:
1. The clinical applicability of the paper would benefit from attempts to correlate this high A20 expression phenotype with clinically relevant AML subsets (risk category, genotype) or even with proposed induction failure prediction scores (Herod et al. Haematologica. 2018; 103(3): 456–465), though this may not be possible given small N in each of these subtypes.
2. While A20 seems to be associated with risk and resistance in AML, years prior, it was illustrated to be ISR gene associated with anti-inflammatory phenotype in the endothelium (Daniel et al. Transplant Proc. 2006 Dec;38(10):3225-7), and protective in transplant vasculopathy. In addition, necroptosis seems to be a feed-forward phenomenon in AML precursor states (eg MDS, Wagner, et al. Blood. 2019. 10;133(2):107-120), so inhibition of necroptosis would, perhaps, be very context dependent. How does this query mesh with the authors' claims? Perhaps this can be discussed in the manuscript more fully.
3. Figure 1H. The western blot is used to make the claim that A20 protein levels are higher in AML cell lines than healthy human tissue, however, the GADPH band is so weak in the healthy controls that it is hard to make the conclusion that they have less A20 expression

and a subsequent figure (2H) shows a strong A20 band in normal CD34 cells. Lower A20 mRNA levels are clearly shown elsewhere and functional knockdown studies with healthy cells in subsequent figures do still make a clear case that healthy cells are less A20 dependent for proliferation (2I). However, I would recommend strengthening the data that healthy human CD34 cells have lower A20 protein levels (eg repeating western blots, proteomics data sets).
4. Figure 2B & 6E. While the trend clearly shows increased doxo-resistance with high A20, the doses used (500nM and 2.5nM respectively) are both well below clinically relevant concentrations seen in patients (~6uM - Clin Cancer Res. 2017 Jul 15; 23(14): 3489–3498) and higher doses begin to overcome this phenomenon in these models. This leaves open the possibility that at relevant clinical doses, the correlation between high A20 expression and doxorubicin resistance could be less relevant. The outcomes data from public data sets and mouse models are helpful in overcoming this limitation, yet the question is not fully answered in the text.
5. Necroptosis is inherently an inflammatory cell-death process, and the immune bone marrow micro-environment has been implicated to be highly relevant. A majority of models used in this paper are in vitro or in immune deficient mice and so are unable to model contributions from immune cells. I would add more language in the discussion section highlighting the lack of investigation of the immune cell contribution as a limitation, and it would strengthen the manuscript to have more relevant models which address this concern.
6. It would also be interesting to see if there is a correlation with circulating or bone marrow DAMPs (i.e. TNFa) with A20 expression, given their critical role in necroptosis.

Minor comments on style, syntax and presentation:
378 – the word mechanism should be plural
414 – there should be commas around the drug name “birinapant”
416 – I would hesitate to say that 7+3 chemotherapy is “not indicated” in poor-risk AML. There are compelling reasons to consider clinical trials or less intensive therapy in this population but induction with 7+3 and stem cell transplant remains a commonly practiced treatment. Perhaps, “growing less popular” or “less effective” etc…

Reviewer #2 (Remarks to the Author):

Summary: In the present study, the authors demonstrate that acute myeloid leukemia (AML) patients with primary resistance to standard 7 +3 induction therapy have elevated expression of NF-kB target genes, with a focus on TNFAIP3/A20. Ultimately, they showed AML specimens with high A20 expression display resistance to anthracyclines, while A20 low samples showed enhanced sensitivity. Loss of A20 expression restored anthracycline sensitivity by inducing necroptosis. Deletion or knockdown of A20 suppressed AML cell growth by inducing necroptosis. They further went on to show that A20 prevents necroptosis by targeting RIPK1. Altogether, their findings suggest that NF-kB-mediated A20 overexpression promotes AML cell growth by impairing necroptosis. This is an interesting story because they are talking about targeting an alternative cell death pathway in AML. The paper is well written and adds novelty to the field. Minor comments to improve the paper

are included below:

1. Please spell out nuclear factor-kappa B upon first usage.
2. Please include a clearly stated hypothesis in the Introduction section.
3. Please include the IKK-Inh doses on Figure 1I-J.
4. Line 133: Please include a sentence that summarizes the results from that section.
5. Supplemental Figure 1 Legend: Please replace k's with a kappa symbol for all instances of NF-kB.
6. Please provide statistics for the data presented in Supplemental Figure 1F.
7. In Figure 2E, the effect of A20 alone on cell counts is not visible with the data normalized as they are. Did A20 alone have any effect on cell counts?
8. Are the authors sure that A20 deletion has no effect on normal primary CD34+ cells? What are the p values for the colony data presented in Figure 2I? There does appear to be an effect, although a therapeutic window may exist.
9. Is A20 upregulated in AML with VEN/AZA resistance? These data might be available from published studies.
10. Figure 1A legend: Please indicate the number of patients included in the RNAseq analysis.
11. It is unclear how many replicates were performed for some of the experiments. Please clarify throughout the manuscript or add information to the Methods section.

Reviewer #3 (Remarks to the Author):

This study aims at elucidating the functional role of A20, a NF-KB target gene, in AML cells maintenance and their chemo-resistance. By employing genetic tool targeting of A20, the authors show that AML survival upon A20 inhibition is impaired likely due to necroptotic cell death. There is also an association between resistance to anthracyclines and ability to induce necroptosis. Overall, the findings described here are novel, important for the field and might be of interest for the broader readership of Nature Communication. However, I found that some concerns should be addressed and preclude publication in its current form. I also believe that the manuscript is easy to read.

Q1: Many previous work has been done to understand conventional chemotherapy resistance, such as SAMHD1 in cytarabine resistance. These facts need to be acknowledged in the introduction.

Q2: While TCGA AML datasets have been datamined, other AML datasets which include a few normal healthy controls should be studied, as it's not clear relative to normal healthy donors, whether A20 levels in AML are higher than those in normal CD34+ cells.

Q3: AML is characterized by cytogenetic abnormalities and mutations, whether A20 expression is related to any particular cytogenetics or mutation?

Q4: In Figure 2H, A20-high (n=3) and A20-low (n=3) have been shown, however, the statistical considerations are not well justified. The results are not sufficient to support the conclusion that A20High AML samples were resistant to Doxorubicin treatment, while A20-low AML were sensitive to Doxorubicin. Moreover, it is not clear why apoptosis assessment was used, as the authors claimed that Doxorubicin resistance is somehow related to necroptosis. Whether the cell death induced by Doxorubicin can be reversed by Nec-1? How much relevant of necroptosis induction has been achieved by Doxorubicin? Ex-vivo culture of primary AML is notoriously difficult. And the different dose of Doxrubicin may induce different type of cell death. These details are fundamental to ensure reproducibility of the findings by other labs.

Q5: Most of the phenotypic studies (Figs3-5) have been performed using MLL-AF9, MN1 AML mouse models, it's not clear how much relevance of necroptosis induction seen after A20 inhibition to primary human AML cells. Some ex-vivo studies will clarify this point.

Q6: GSEA analysis shown in Fig.4B did not support the claim that "Deletion of A20 resulted in a significant enrichment of genes associated with both NF-kB and necroptosis activation as compared to wild-type MLL-AF9 AML cells", as the NES score and p value are not significant.

Q7: Fig.6G not reflect the difference shown in Fig.6H, as the authors claimed that "For example, patient AML329 at diagnosis exhibited 1% of malignant cells expressing A20, which primarily were classified as HSC, progenitor, cDC-like, and monocytic-like cells (Figure 6H). At Day 37 (30 days post induction therapy), >5% of the malignant cells expressed A20". Please clarify.

Point-by-point response

We thank the reviewers and editor for their positive responses and insightful suggestions. We were pleased to see that the reviewers appreciated our work, and we extend our thanks to them for their thorough analysis of the manuscript and their constructive feedback. Overall, we feel that the recommendations have significantly strengthened the manuscript. As indicated by the editorial team, we focused on:

- Examining A20 expression across AML patients and correlating it with mutations and subtypes (reviewers #1, #3).
- Providing additional data on A20 expression in healthy versus AML cells and its relevance in healthy cells (all reviewers).
- Additional validation of the proposed mechanism through necroptosis (reviewers #1, #3).
- Providing additional details on the statistical analyses (reviewers #2, #3).

Below is a summary of the new data:

- We correlated A20 expression with clinical features in AML patients (new **Supplemental Figure 1E**).
- We repeated all immunoblots and now more convincingly show that A20 levels are indeed low in normal CD34+ cells and generally higher in AML cell lines and patient-derived samples (new **Figure 1H** and **Figure 2A**).
- To further strengthen the claim that A20 expression is higher in AML versus healthy cells, we have also provided new data from publicly available datasets (new **Figure 1G**).
- We examined necroptosis and whether Nec-1 (the necroptosis inhibitor) can reverse the observed cell death using PDX-AML samples ($A20^{Low}$ and $A20^{High}$) (new **Figure 6D**).
- To ensure that A20 suppresses necroptosis, we knocked down A20 in $A20^{High}$ PDX-AML and found a significant increase in necroptosis markers (new **Supplemental Figure 5C**).
- We examined whether A20 plays a role at higher doxorubicin concentrations. We performed new experiments demonstrating that: (1) Treatment of AML cells with higher concentrations of doxorubicin selected for cells with higher levels of A20 protein (**Supplemental Figure 7C**). (2) Overexpression of A20 in AML cells resulted in decreased sensitivity to doxorubicin even at higher concentrations (**Supplemental Figure 7E**).

Overall, our revised manuscript provides additional evidence that A20 is a gatekeeper in preventing necroptotic cell death in AML and a barrier to chemotherapy responses. Moreover, our findings establish that a non-canonical programmed cell death mechanism drives chemotherapy responses in AML. These insights into the underlying mechanisms of chemotherapy induction failure will inform new therapeutic targets, such as A20, and clinical trials to counteract the escape of chemotherapy-resistant AML clones.

Reviewer #1

In this manuscript, the Starczynowski laboratory identifies A20 as an important downstream effector of chemoresistance in AML. The authors present data that proposes A20 induced NF-kB expression leading to activation of a chemo-resistance gene network highlighted by A20's ability to impair necroptotic cell death. Their hypothesis was generated by gene expression analysis of publicly available AML data sets with supportive functional studies in both human AML cell lines, primary AML patient samples and A20 knock-in and knock-down mouse models. They elevate their work by conducting thorough mechanistic studies by combining their A20 knock-out model with chemical and genetic inhibition of various hypothesized effectors of A20 pro-leukemia function, coming to the conclusion that A20 promotes leukemia growth and doxorubicin resistance by suppressing RIPK3-mediated necroptosis through its E3-ligase function rather than through mechanisms involving its negative-feedback on NF-kB.

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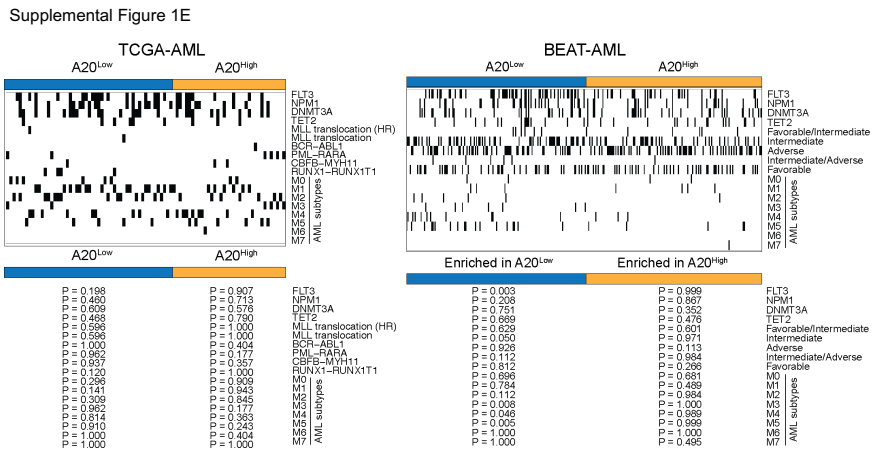
RESPONSE: We thank the reviewer (along with Reviewer #3) for making this recommendation. To determine whether A20 overexpression correlates with specific clinical features in AML patients, we



Supplemental Figure 1E. Mutations or AML subtype in patients with AML from TCGA (left) or BEAT-AML (right) clustered in Group 1 (low A20 expression) and Group 2 (high A20 expression). P values were determined with hypergeometric testing.

stratified AML patients based on high (Z score >1.5) or low A20 expression (Z score < 1.5). AML patients with elevated A20 expression were not enriched for any of the common mutations or subtypes at diagnosis (**Supplemental Figure 1E**). We attempted to correlate induction failure prediction scores, but our numbers were too small to yield a meaningful conclusion.

2. While A20 seems to be associated with risk and resistance in AML, years prior, it was illustrated to be ISR gene associated with anti-inflammatory phenotype in the endothelium (Daniel et al. Transplant Proc. 2006 Dec;38(10):3225-7), and protective in transplant vasculopathy. In addition, necroptosis seems to be a feed-forward phenomenon in AML precursor states (eg MDS, Wagner, et al. Blood. 2019. 10;133(2):107-120), so inhibition of necroptosis would, perhaps, be very context dependent. How does this query mesh with the authors' claims? Perhaps this can be discussed in the manuscript more fully.

RESPONSE: The reviewer raises important points, which we have now reviewed and discussed the context-dependent role of necroptosis in myeloid malignancies (see pages 15-16). As noted by the reviewer, necroptosis has also been studied in MDS. It has been shown that the necroptotic pathway may contribute to the pathogenesis of MDS by promoting excessive cell death of maturing hematopoietic cells and inflammation in the BM (Wagner et al., Blood 2019). The observed necroptosis in MDS may be due to loss of A20 as a recent paper revealed that A20 levels are lower in mature blood cells from MDS patients (Wang et al., Hematology, 2024). However, A20 levels are higher in pre-leukemic and MDS HSPCs (Muto et al., Nature Immunology, 2020 and Jakobsen et al., Cell Stem Cell, 2024). Based on these findings along with our new data, we posit that in AML, the leukemic cells have subverted the ability to activate the necroptosis pathways, such as through elevated expression of A20. Thus, targeting necroptosis with small molecule modulators is emerging as a new approach in cancer therapy, offering the advantage of bypassing apoptosis resistance.

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RESPONSE: We apologize that the quality of the original immunoblots were difficult to interpret. We have repeated all of the immunoblots and now more convincingly show that A20 levels are indeed low in normal CD34+ cells and generally higher in AML cell lines and patient-derived samples (**Figure 1H** and **Figure 2A**). To further strengthen the claim that A20 expression is higher in AML vs healthy cells, we have provided new data from publicly available data sets (see **Figure 1G**). Collectively, the prior and new data support the claim that A20 is elevated in a subset of AML as compared to normal/healthy hematopoietic cells.

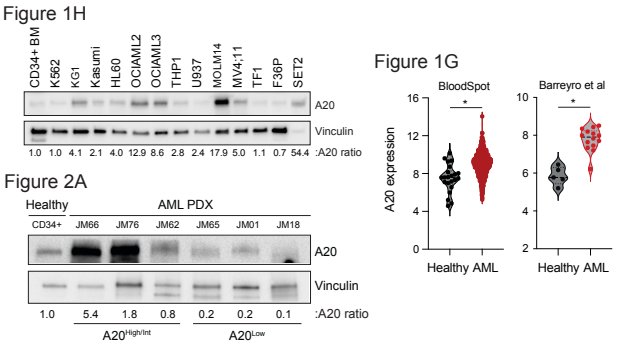


Figure 1G. A20 expression in AML patient blasts versus healthy BM cells (BEAT-AML) or in AML GMPs versus healthy control GMPs (GSE35008 and GSE35010). **Figure 1H,** Immunoblot showing A20 protein expression in healthy human cells and a panel of human AML cell lines. Relative A20 protein was normalized to Vinculin and set at 1.0 to CD34+ BM cells. **Figure 2A.** Immunoblot of A20 protein in a panel of AML patient-derived xenografts (PDX).

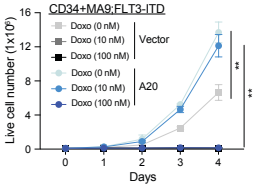
4. Figure 2B & 6E. While the trend clearly shows increased doxo-resistance with high A20, the doses used (500nM and 2.5nM respectively) are both well below clinically relevant concentrations seen in patients (~6uM - Clin Cancer Res. 2017 Jul 15; 23(14): 3489–3498) and higher doses begin to

overcome this phenomenon in these models. This leaves open the possibility that at relevant clinical doses, the correlation between high A20 expression and doxorubicin resistance could be less relevant. The outcomes data from public data sets and mouse models are helpful in overcoming this limitation, yet the question is not fully answered in the text.

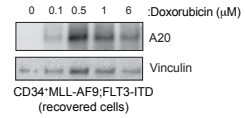
RESPONSE: The reviewer raises an important point about the pharmacokinetic properties of doxorubicin, which we inadvertently did not address in the original submission. Based on published studies (Clin Cancer Res), we evaluated the clinically relevant concentrations of doxorubicin, taking into consideration the Cmax, the dosing schedule for AML (45 mg/kg daily), half-life in plasma, and protein binding/free drug levels (see Clin Cancer Res, 2017). Based on these parameters, the approximate free drug concentrations range from ~100 to 1000 nM when considering Cmax and its half-life. Prior studies have used a wide range of doxorubicin concentrations (low nanomolar to micromolar), but most studies commonly examine doxorubicin at < 1 μM. Thus, the free drug exposure levels in patients are generally consistent with the concentrations used in our study. Each cell line had a different sensitivity to doxorubicin, so we adjusted the concentrations accordingly. One human-derived sample, CD34+ cells expressing FLT3-ITD and MLL-AF9, was exquisitely sensitive to doxorubicin. In the revised manuscript, we provide a rationale for the doxorubicin concentrations used in our experiments.

Since necroptosis is thought to be induced at higher chemotherapy exposures as a backup mechanism of cell death to apoptosis, we also examined whether A20 plays a role at higher doxorubicin concentrations. For this, we performed new experiments which demonstrated that: 1) treatment of AML cells with higher concentrations of doxorubicin selected for cells with higher levels of A20 protein (**Supplemental Figure 7C**); 2) overexpression of A20 in AML cells resulted in increased resistance to doxorubicin even at higher concentrations (**Supplemental Figure 7E**).

Supplemental Figure 7C



Supplemental Figure 7E



Supplemental Figure 7C. Proliferation assay showing live cells by trypan blue exclusion in CD34+ MLL-AF9;FLT3-ITD cells treated with the indicated concentrations of doxorubicin. **Supplemental Figure 7E.** A20 protein expression was evaluated on CD34+ MLL-AF9;FLT3-ITD cells recovered following 5 day treatment with the indicated concentrations of doxorubicin.

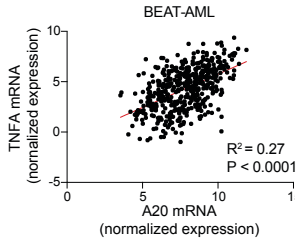
5. Necroptosis is inherently an inflammatory cell-death process, and the immune bone marrow micro-environment has been implicated to be highly relevant. A majority of models used in this paper are in vitro or in immune deficient mice and so are unable to model contributions from immune cells. I would add more language in the discussion section highlighting the lack of investigation of the immune cell contribution as a limitation, and it would strengthen the manuscript to have more relevant models which address this concern.

RESPONSE: Thank you for the suggestions. We provide a discussion on the relevance of the immune microenvironment and state that future studies should consider the crosstalk between the microenvironment and necroptosis regulation. Please refer to page 14.

6. It would also be interesting to see if there is a correlation with circulating or bone marrow DAMPs (i.e. TNFa) with A20 expression, given their critical role in necroptosis.

RESPONSE: The expression of TNF and A20 was correlated in AML patient samples. Interestingly, we observed a positive correlation in TNF and A20 expression in AML samples (**Supplemental Figure 5A**). We also discussed the implications of this observation in the discussion (Page 14).

Supplemental Figure 5A



Supplemental Figure 5A. Correlation of TNFa and A20 mRNA expression in primary AML samples (adapted from BEAT-AML dataset).

Minor comments on style, syntax and presentation:

378 – the word mechanism should be plural

RESPONSE: Corrected.

414 – there should be commas around the drug name “birinapant”

RESPONSE: Corrected.

416 – I would hesitate to say that 7+3 chemotherapy is “not indicated” in poor-risk AML. There are compelling reasons to consider clinical trials or less intensive therapy in this population but induction with 7+3 and stem cell transplant remains a commonly practiced treatment. Perhaps, “growing less popular” or “less effective” etc.

RESPONSE: Corrected.

Reviewer #2

Summary: In the present study, the authors demonstrate that acute myeloid leukemia (AML) patients with primary resistance to standard 7 +3 induction therapy have elevated expression of NF-kB target genes, with a focus on TNFAIP3/A20. Ultimately, they showed AML specimens with high A20 expression display resistance to anthracyclines, while A20 low samples showed enhanced sensitivity. Loss of A20 expression restored anthracycline sensitivity by inducing necroptosis. Deletion or knockdown of A20 suppressed AML cell growth by inducing necroptosis. They further went on to show that A20 prevents necroptosis by targeting RIPK1. Altogether, their findings suggest that NF-kB-mediated A20 overexpression promotes AML cell growth by impairing necroptosis. This is an interesting story because they are talking about targeting an alternative cell death pathway in AML. The paper is well written and adds novelty to the field. Minor comments to improve the paper are included below:

1. Please spell out nuclear factor-kappa B upon first usage.

RESPONSE: Corrected.

2. Please include a clearly stated hypothesis in the Introduction section.

RESPONSE: A hypothesis is included in the introduction (Page 4): "*Given the pleiotropic role of NF-κB, we posited that increased NF-κB signaling, through specific target genes, results in diminished chemotherapy responses in AML*".

3. Please include the IKK-Inh doses on Figure 1I-J.

RESPONSE: Information provided (see **Figure 1I-J**).

4. Line 133: Please include a sentence that summarizes the results from that section.

RESPONSE: A summary statement is included (Page 5): "*Collectively, we found that patients who failed to achieve complete response after induction chemotherapy had enriched NF-kB signaling pathways, particularly the gene TNFAIP3 (A20), which was associated with higher induction failure rates and worse overall survival compared to those who initially responded to therapy*."

5. Supplemental Figure 1 Legend: Please replace k's with a kappa symbol for all instances of NF-kB.

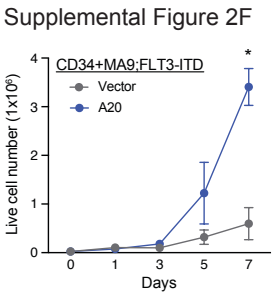
RESPONSE: We have corrected the kappa symbol in all cases.

6. Please provide statistics for the data presented in Supplemental Figure 1F.

RESPONSE: The statistics are now provided in the new **Supplemental Figure 2**. In the revised manuscript, we also evaluated the correlation between doxorubicin response and A20 expression in primary patient samples in several ways: 1) A20 expression was compared between AML samples that were deemed sensitive or resistant to doxorubicin, which showed that A20 was higher in more resistant samples (original analysis: **Supplemental Figure 2B**)(unpaired two-tailed T test, P = 0.1); 2) We performed a correlation analysis and observed that A20 expression positively correlated with AML cell viability following doxorubicin treatment (new **Supplemental Figure 2C**)(R^2 = 0.13); 3) The relative inhibition of cell viability by doxorubicin was lower in $A20^{High}$ AML samples (**Supplemental Figure 2D**)(P = 0.09). Given the small data set and variability among primary patient samples, we didn't achieve statistical significance, however, the overall trend along with our new validation studies strongly suggests that A20 expression drives doxorubicin sensitivity and responses in AML.

7. In Figure 2E, the effect of A20 alone on cell counts is not visible with the data normalized as they are. Did A20 alone have any effect on cell counts?

RESPONSE: The effects of A20 overexpression on AML cells are now included in **Supplemental Figure 2F**. Consistent with our claims that A20 is required for AML, this new data shows that elevated A20 expression promotes increased proliferation of human CD34+ MLL-AF9;FLT3-ITD AML cells as compared to vector-expressing cells.

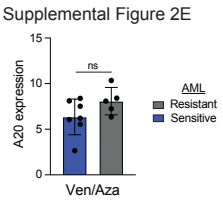


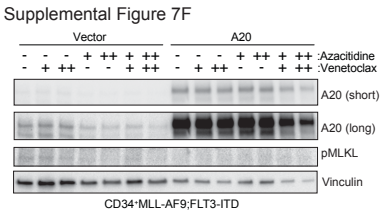
Supplemental Figure 2F. Cell viability (trypan blue exclusion) of MLL-AF9;FLT3-ITD cells expressing an empty vector or A20 following treatment with doxorubicin.

8. Are the authors sure that A20 deletion has no effect on normal primary CD34+ cells? What are the p values for the colony data presented in Figure 2I? There does appear to be an effect, although a therapeutic window may exist.

RESPONSE: We apologize for the inadvertent omission of the P values in Figure 2I. The P values are now included for all figures. We agree with the author's assessment of the data. Knockdown of A20 in normal CD34+ cells results in a modest reduction in colony formation, but not to the extent observed in AML cells (**Figure 2J**). A similar effect was observed in mouse HSPCs (**Figure 3C**). We rephrased our conclusion to reflect the findings that knockdown of A20 in healthy hematopoietic cells has a modest effect on colony-forming progenitor (see page 7).

9. Is A20 upregulated in AML with VEN/AZA resistance? These data might be available from published studies.





Supplemental Figure 2E. TNFAIP3/A20 expression in AML PDX samples that are either resistant or sensitive to venetoclax (Ven) and azacitidine (Aza) (adapted from Pei et al, Cancer Discovery, 2020). **Supplemental Figure 7F.** A20 protein expression was evaluated in CD34+ MLL-AF9;FLT3-ITD cells expressed vector or A20 following treatment with azacitine or venetoclax (+, low; ++, high).

RESPONSE: The authors raise an interesting question. As suggested, we evaluated AML samples that are VEN/AZA sensitive or resistant and examined A20 expression at diagnosis (data from Pei et al, Cancer Discovery, 2020). The expression of A20 was not different between the sensitive and resistant groups at diagnosis (**Supplemental Figure 2E**). In addition, we examined whether VEN/AZA treatment results in increased A20 expression. As shown in **Supplemental Figure 7F**, we did not see an increase in A20 expression following VEN/AZA treatment. In contrast, A20 expression increased in a dose-dependent manner following doxorubicin treatment (**Figure 6A,B**). These data suggest that overexpression of A20 is primarily observed in AMLs that are resistant to anthracyclines (i.e., doxorubicin).

10. Figure 1A legend: Please indicate the number of patients included in the RNAseq analysis.

RESPONSE: The number of patients is now included in the results (Page 4) and figure captions (Page 24).

11. It is unclear how many replicates were performed for some of the experiments. Please clarify throughout the manuscript or add information to the Methods section.

RESPONSE: Information on replicates is now included for all experiments. See figure legends. The source data is also included for all experiments (see "**Source Data**" file).

Reviewer #3

This study aims at elucidating the functional role of A20, a NF-KB target gene, in AML cells maintenance and their chemo-resistance. By employing genetic tool targeting of A20, the authors show that AML survival upon A20 inhibition is impaired likely due to necroptotic cell death. There is also an association between resistance to anthracyclines and ability to induce necroptosis. Overall, the findings described here are novel, important for the field and might be of interest for the broader readership of Nature Communication. However, I found that some concerns should be addressed and preclude publication in its current form. I also believe that the manuscript is easy to read.

Q1: Many previous work has been done to understand conventional chemotherapy resistance, such as SAMHD1 in cytarabine resistance. These facts need to be acknowledged in the introduction.

RESPONSE: We agree with the reviewer and have now expanded the introduction to discuss additional prior work on chemotherapy resistance in AML. See page 3.

Q2: While TCGA AML datasets have been datamined, other AML datasets which include a few normal healthy controls should be studied, as it's not clear relative to normal healthy donors, whether A20 levels in AML are higher than those in normal CD34+ cells.

RESPONSE: We have repeated all of the immunoblots and now more convincingly show that A20 levels are indeed low in normal CD34+ cells and generally higher in AML cell lines and patient-derived samples (**Figure 1H** and **Figure 2A**). To further strengthen the claim that A20 expression is higher in AML vs healthy cells, we have provided new data from publicly available data sets (see **Figure 1G**). Collectively, the prior and new data support the claim that A20 is elevated in a subset of AML as compared to normal/healthy hematopoietic cells.

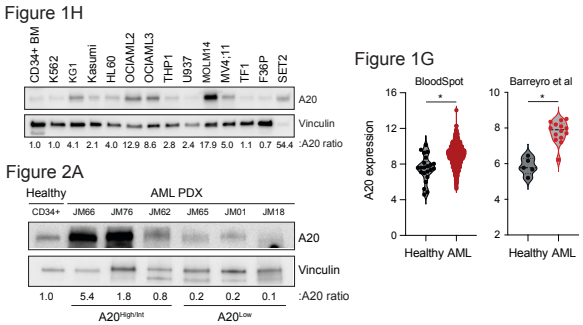
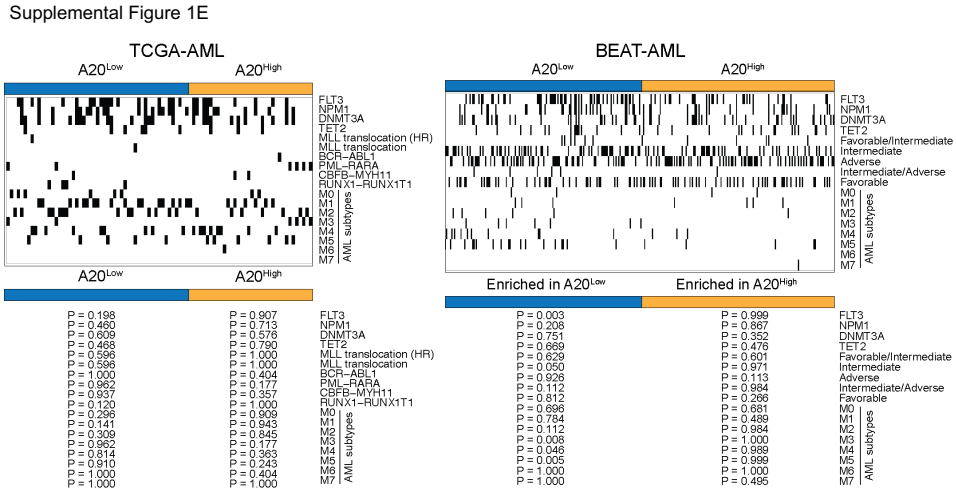


Figure 1G. A20 expression in AML patient blasts versus healthy BM cells (BEAT-AML) or in AML GMPs versus healthy control GMPs (GSE35008 and GSE35010). **Figure 1H.** Immunoblot showing A20 protein expression in healthy human cells and a panel of human AML cell lines. Relative A20 protein was normalized to Vinculin and set at 1.0 to CD34+ BM cells. **Figure 2A.** Immunoblot of A20 protein in a panel of AML patient-derived xenografts (PDX).

Q3: AML is characterized by cytogenetic abnormalities and mutations, whether A20 expression is related to any particular cytogenetics or mutation?

RESPONSE: We thank the reviewer (along with Reviewer #1) for making this recommendation. To determine whether A20 overexpression correlates with specific clinical features in AML patients, we stratified AML patients based on high (Z score >1.5) or low A20 expression (Z score < 1.5). AML patients with elevated A20 expression were not enriched for any of the common mutations or subtypes at diagnosis (**Supplemental Figure 1E**).



Supplemental Figure 1E. Mutations or AML subtype in patients with AML from TCGA (left) or BEAT-AML (right) clustered in Group 1 (low A20 expression) and Group 2 (high A20 expression). P values were determined with hypergeometric testing.

Q4: In Figure 2H, A20-high (n=3) and A20-low (n=3) have been shown, however, the statistical considerations are not well justified. The results are not sufficient to support the conclusion that A20High AML samples were resistant to Doxorubicin treatment, while A20-low AML were sensitive to Doxorubicin.

RESPONSE: We apologize for the insufficient description of our statistical analysis for Figure 2H. We have now updated the figure to better illustrate the data analysis and statistical comparisons. Briefly, the comparisons were between the doxorubicin-treated and untreated groups (normalized to 1.0) for individual patient samples (new **Figure 2B**). In addition, we examined the significance between the highest doxorubicin treatment group (500 nM) for the $A20^{Low}$ and $A20^{High/Int}$ samples (see new **Figure 2C**). This new data demonstrates that elevated levels of A20 in AML confer reduced sensitivity to doxorubicin. We feel that the revised data better supports our conclusion that the $A20^{High/Int}$ AML samples are intrinsically more resistant to doxorubicin treatment.

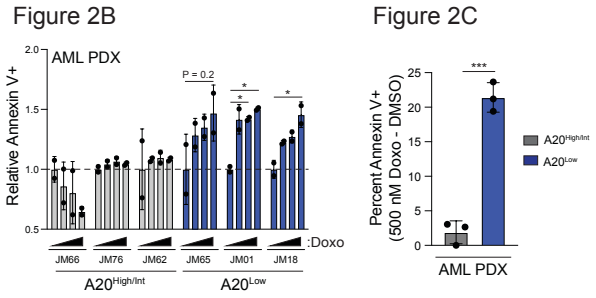


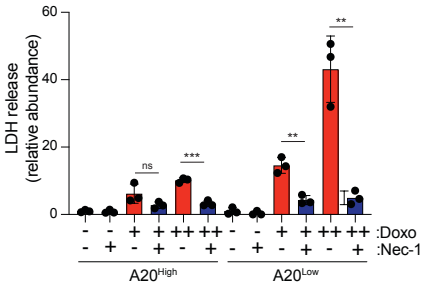
Figure 2B. Flow cytometry analysis of Annexin V positive AML PDX cells following treatment with increasing doses of doxorubicin (0, 100, 250, 500 nM) for 24 and 48 hours. **Figure 2C.** Percent of Annexin V positive cells following treatment with 500 nM doxorubicin in $A20^{High/Int}$ and $A20^{Low}$ AML PDX cells (calculated from panel B). Dots represent individual patients.

Moreover, it is not clear why apoptosis assessment was used, as the authors claimed that Doxorubicin resistance is somehow related to necroptosis. Whether the cell death induced by Doxorubicin can be reversed by Nec-1? How much relevant of necroptosis induction has been achieved by Doxorubicin? Ex-vivo culture of primary AML is notoriously difficult. And the different dose of Doxrubicin may induce different type of cell death. These details are fundamental to ensure reproducibility of the findings by other labs.

RESPONSE: Although there are many assays for detection of apoptosis, relatively few assays are available for measuring necrosis. A key signature for necrotic cells is the permeabilization of plasma membrane. This event can be quantified in tissue culture settings by measuring the release of the enzyme lactate dehydrogenase (LDH). When combined with other methods, measuring LDH release is a useful method for detection of necrosis. (Brauchle et al., 2014 Scientific Reports). In the initial assessment of AML cell sensitivity to doxorubicin, we utilized AnnexinV staining alone as it can capture both late-apoptosis and primary necrosis (Crowley et al., Cold Spring Harb Protoc, 2016). We address this in the results section (see page 6). To determine the relevance of necroptosis in doxorubicin-mediated cell death in AML, we examined necroptosis using LDH staining and also whether Nec-1 can reverse the observed cell death using two PDX-AML samples ($A20^{Low}$ and $A20^{High}$). As shown in (new) **Figure 6D**, doxorubicin (at low [+] and high [++] concentrations) results in LDH release in the $A20^{Low}$ AML, which can be nearly completely reversed with Nec-1 treatment. In contrast, doxorubicin was less efficient at inducing necroptosis (i.e., LDH release) in the $A20^{High}$ AML sample (**Figure 6D**).

To ensure that A20 suppresses necroptosis, we knocked down A20 in the $A20^{High}$ PDX-AML and found a significant increase in LDH release (**Supplemental Figure 5C**), suggesting that A20 prevents necroptosis in AML. We agree with the reviewer that doxorubicin can induce both apoptosis and necroptosis in AML, however, our data suggests that the primary therapeutic mechanism of action is related to its ability to induce necroptosis. These points are now discussed in the discussion section (pages 15-16).

Figure 6D



Supplemental Figure 5C

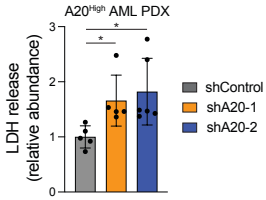


Figure 6D. LDH release assay of an $A20^{High}$ (JM62) and $A20^{Low}$ (JM18) PDX AML samples treated with doxorubicin (+, 250 nM; ++, 500 nM) and/or Necrostatin-1 (Nec-1, 30 μM). n = 2 independent technical replicates. **Supplemental Figure 5C.** Necroptosis was assayed in $A20^{High}$ AML patient-derived samples (JM62) using the lactate dehydrogenase (LDH) release assay.

Q5: Most of the phenotypic studies (Figs3-5) have been performed using MLL-AF9, MN1 AML mouse models, it's not clear how much relevance of necroptosis induction seen after A20 inhibition to primary human AML cells. Some ex-vivo studies will clarify this point.

RESPONSE: We thank the reviewer for requesting important ex vivo studies in PDX-AML cells to strengthen the connection to necroptosis. As outlined in more detail in point #4, we have provided additional evidence that necroptosis is operational in primary AML: (1) To determine the relevance of necroptosis to doxorubicin-mediated cell death in AML, we examined necroptosis using LDH staining and also whether Nec-1 can reverse the observed cell death using two PDX-AML samples ($A20^{Low}$ and $A20^{High}$). As shown in **Figure 6D**, doxorubicin (at low and high concentrations) results in LDH release (a marker of necroptosis) in the $A20^{Low}$ AML, which can be nearly completely reversed with Nec-1 treatment. In contrast, doxorubicin was less efficient at inducing necroptosis (i.e., LDH release) in the $A20^{High}$ AML sample (**Figure 6D**). (2) To ensure that A20 suppresses necroptosis, we knocked down A20 in the $A20^{High}$ PDX-AML and found a significant increase in LDH release (**Supplemental Figure 5C**), suggesting that A20 prevents necroptosis in AML.

Q6: GSEA analysis shown in Fig.4B did not support the claim that "Deletion of A20 resulted in a significant enrichment of genes associated with both NF-kB and necroptosis activation as compared to wild-type MLL-AF9 AML cells", as the NES score and p value are not significant.

RESPONSE: We thank the reviewer for this point. We agree that the enrichment of NF-kB genes is not highly significant. However, we do observe activation of NF-kB signaling in A20-deficient AML cells as

measured by immunoblotting for phospho-IKKb (see **Supplemental Figure 5D**). The claim of our finding is that loss of A20 can lead to NF-kB activation, but NF-kB is not responsible for the phenotype (see **Figures 4D-F** and **Supplemental Figure 6B).** Necroptosis signatures are poorly defined, so we are not surprised that the enrichment score was not highly significant. Nevertheless, deletion of A20 in AML cells resulted in robust necroptosis (confirmed by various molecular and cellular measures), which was responsible for the suppression of the leukemic cells (see **Figures 4D-I**). As such, we have clarified these points in the results section (see page 9).

Q7: Fig.6G not reflect the difference shown in Fig.6H, as the authors claimed that “For example, patient AML329 at diagnosis exhibited 1% of malignant cells expressing A20, which primarily were classified as HSC, progenitor, cDC-like, and monocytic-like cells (Figure 6H). At Day 37 (30 days post induction therapy), >5% of the malignant cells expressed A20”. Please clarify.

RESPONSE: We apologize for not providing sufficient clarity for the data in Figures 6G,H. In the revised manuscript, we provide a detailed explanation of the analysis along with new versions of the figure (**Figure 6H,I**).

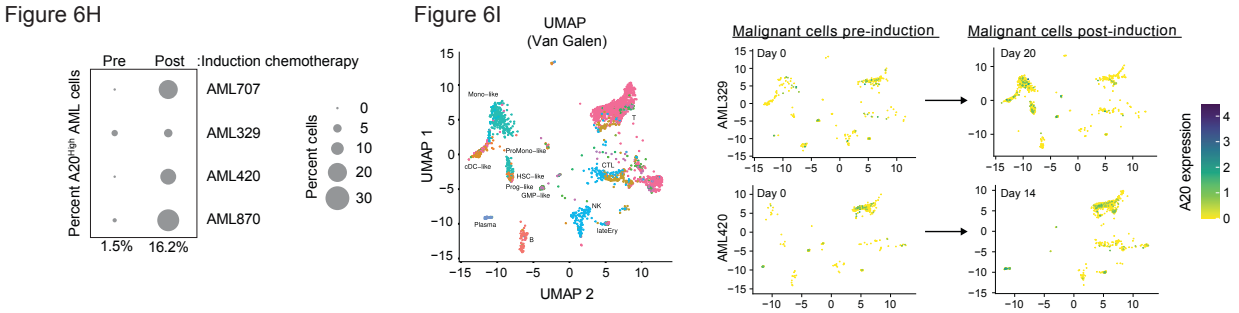


Figure 6H. Percent of AML cells with A20 overexpression (Z score > 0.25) pre- and post-chemotherapy. **Figure 6I.** UMAP plots of single-cell RNA sequencing data (GSE116256) showing AML cell fate mapping (left), AML cells prior to chemotherapy in patient AML329 and AML420, and AML cells post-chemotherapy (right).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have thoughtful resolved outstanding queries, and significantly improved upon their initial manuscript. I have no further requests or outstanding questions.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my concerns.

Reviewer #3 (Remarks to the Author):

The manuscript revised has addressed most of my comments, I just have one minor comment of Figure 6I of AML420, if the authors can explain why the gene expression of A20 is upregulated in T cell or CTL subset, the HSCs part has no events shown.