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Research Plan

Category: Cellular and Molecular Biology

Title: Targeted inhibition of a novel MALT1 and MAPK signaling network synergistically suppresses aggressive B cell lymphoma growth

With the approvement of ibrutinib, a specific inhibitor which blocks the BCR/TLR pathway by inhibiting the commonly mutated and an upstream protein BTK in the activated B cell subtype of Diffuse large B cell lymphoma [1], targeted therapy has become the blossoming field of current and future intruinings. While ABC DLBCL is characterized by hallmark of mutations leading to the Nuclear factor kappa B activation (Nf-kB) [2,3] one of the pathways that is downstream of the BTK is functional due to a multimeric complex between CARD11, BCL10, MALT1 [3,4]. Furthermore, MALT1 – Muscoa associated lymphoid translocation protein 1- is mutated in 70% of ABC DLBCL cases [4] and its inhibition is fatal to ABC DLBCL cell lines *in vitro, in vivo and ex vivo* [5,6]. MALT1 functions as the only human paracaspase: it has scaffolding function which allows it to recruit kinases for supporting Nf-kB nuclear translocation [7] but then it also has a protease domain, which allows it to cleave negative regulaors of the Nf-kB pathway [7]– tumor suppressor – including Regnase 1, LIMA, HOIP, and A20. In this regard, Fontan et al. 2018 recently reported a specific inhibitor of the MALT1 protease which covalently binds it and attenuates it proteyolic activity. Moreover, they reported that at 48hrs and beyond MALT1 expression seems to increase back. Thus, this study is designed to define the biological mechanisms involved in increase of MALT1 expression, which certainly can be a major underlying cause of resistance which 40% of the patients face. Our approach is based on the idea of a feedback loop that contributes to MALT1 expression; we'll be employing a combination of SCM-02-138 and trametinib, which inhbits MEK kinase and is downstream of BTK as well. **Purpose/Hypothesis**

The purpose of this study is to:

1. Define the mechanism(s) underlying the increase in MALT1 expression at 48hrs in response to specific inhibition
2. Determine if there lies a feedback loop with another protein that causes MALT1 to increase

Correspondingly, it is hypothesized that:

1. MALT1 increase in expression is a direct result of another protein contributing to its activity – essentially feed back loop that increases MALT1 expression
2. MEK kinase contributes to MALT1 activating the feedback loop downstream of MALT1 which causes uptake in MALT1 expression

Methodology: We plan on utilizing ABC DLBCL cell line, isolated from humans by mskcc. Our objective is to combine MALT1 inhibition with MEK inhibition and analyze MALT1 gene expression as well as at the protein level

Role of Mentor: Mentor is essentially responsible for teaching the techniques, guiding thorough the experiments, and particularly helping to troubleshoot.

Role of Student: Student's role is to work through the experiments and obtain data. It is under the essential role of student to not utilize only the supervised equipments and only work through the experiments, priliminarly discussed with the mentor.

Procedures:

Cell lines - HBL-1 cell line is recognized as the universally used cell line for the ABC subtype of DLBCL because of having mutations in CARD11, BCL10, MALT1, as well as in CD79a/b.

- HBL1 employed will be of human tissues derived by mskcc.

IMDM - cell media - will be prepared using 10% Fetal Bovine Serum (FBS) mixed with Lglutamine.

- HBL1 cells will be kept in IMDM at day 0 without any treatment.
- At day, cells will be counted using the chamber counting device - hemocytometer.
- 9 million cells in 18 ml IMDM will be placed in 4 different 25 ml cell culture flasks.

Drug Treatment

Vehicle Cells will be treated with DMSO. But since DMSO can also have its own effects on cells, the above concentrations of drugs will be made in DMSO.

SCM-02-138 will be used to (i) inhibit MALT1 expression for earlier time points and (ii) to further show whether MALT1 expression increases upon specific and covalent inhibition as well.

- One flask will be treated with 1uM of SCM-02-138

Trametinib is an FDA approved drug for MEK inhibition in Melanoma cell lines. However, it hasn't been previously applied in ABC DLBCL cell lines, but since it is a highly specific MEK kinase inhibitor it will be employed

- Trametinib concentration will be 100 nM

Combination (SCM-02-138 + Trametinib) Using combination of MALT1 inhibitor and MEK inhibition, changes in MALT1 expression will be compared to those of solely of MALT1 inhibition.

- Cells (in 6ml) will be collected at 4 hrs, 24 hrs, and 48 hrs each, along with being counted using the hemocytometer.

- Cells in DPBS (1x) will be centrifuged at 300 g for 4 minutes followed by aspiration of the DPBS and the transfer of cell palete to the eppendorf tubes.
- Cells in 4ml of media will then be lysed with the RIPA buffer along with the 2ox protease inhibitor and the 40x protease inhibitor, while being kept on ice for 30 minutes. Whereas the remaining in 2ml will be dissolved in Trizol and stored at -80C.
- Cells, alongside the lysing solution will be centrifuged at **4** 14000 rpm for 3o minutes and the palette, containing the protein will be transferred to new tubes.

Protein Quantification

- A 96 well plate will be utilized; the BSA will be used to get the standard curve for comparisons to the actual protein, through the following concentrations.

Concentration (mg/ml)	BSA (ul)	Water (ul)
0	0.0	10.00
.125	0.063	9.38
.25	1.25	8.75
.5	2.50	7.50

.75	3.75	6.25
1	5.0	5.00
1.5	7.50	2.50

- 250 uL of the protein quantification dye will be added to the wells and later 5ul of the protein solution of the standard as well as the actual protein concentration and three separate wells of 7oul will be created.
- Gen 5 program will then be used to measure the protein at 750nm to achieve the results which will be compared to the curve using Excel.
- Proteins at this this time will either be stored at -80 if the SDS gels not be ready. However, if the SDS gels are pre-made, they'll be treated with the 5x loading buffer and later cooked at 95 for 5min.
- 30ug of each protein will be laded to the gels, while 5ul of the Protein ladder – the marker – will be loaded on both sides. The covered surrounding of the gels will have running buffer of 1:10 with distilled water.
- The proteins will run with the help of the electricity for at least 1.5 hrs or when the marker sign appears to be getting out of the gel.
- Gels will then be transferred to the cold room, where the proteins will be transferred to the membrane at 300 V for 1.5hrs.
- The transfer buffer used be a combination of methanol, transfer buffer, and the water; the concentrations of methanol and the transfer buffer will be 4:10 and 2:10 respectively.
- After the transfer, membranes will be acquired and blotted with the 5% milk for 30 min.
- They will be washed with the PBST buffer for 45 mins, constituting of three 15 minute drills.
- They will then be cut as following:
    - MALT1 has the molecular weight of 92 kDa and pERK has molecular weight of 42kDa, thus the cut will me made at 75kDa.
- Membranes will be cut with the anti-mouse MALT1 antibody corresponding to _____ and the anti-rabbit pERK antibody, corresponding to the ____ epitope.
- They will be incubated overnight, shaking at the in the cold room.
- Next day, membranes will be washed with the PBST buffer for 45min.

- They will then be incubated with the secondary anti-mouse HRP-conjugated antibody for MALT1 and anti-rabbit HRP conjugated antibody for pERK. The secondary antibodies will be made in 5% milk with the dilution of 1:2000. The membranes will be incubated for 2hrs at at room temperature.
- After 2hrs, membranes will be washed with the PBST for 45mins.
- They will then be incubated with the pierce ECL plus solution for 5-10 mins and then viewed under the Bio-Rad's western blotting machine.
- Membranes will then be incubated with the loading control – Actin as following:
- They will be incubated with the stripping buffer for 15min and then washed with the PBST for 20 mins.
- Incubation with the 5% milk for 30mins and 15 minutes PBST wash will then be followed as well.
- Membranes will then be incubated with anti HRP conjugated Actin antibody for 30mins and washed with the PBST for 45 mins. They will then be viewed under the biorad machine using the same Pierce ECL plus solution.

MALT1 expression at the gene level

- Cells in 2ml of media, dissolved in trizol, will be taken out of -80 and vortexed to make them homogenous and kept on ice.
- They will then be treated with 200ul of chloroform and incubated on ice for 15mins and then centrifuged at 1,200 g for 15mins at 4C.
- RNA will be precipitated with 0.5 ml isopropanol and later washed with cold 70% ethanol.
- Centrifugation for 10 minutes at 7500 g, 4C will be followed by the essential step of air drying.
- RNA pallet will then be diluted 1:40 in the RNAase free water.
- RNA concentration will be determined by measuring the absorbance at 260 nm, where nanopore water will be used as a control. Further experiment will only be followed if the 260/280 ratio ends be at least 1.8, otherwise the RNA could had be degraded.

cDNA synthesis

- In a 0.2ml PCR tube, RNA (200ng-500ng) in ul, the selected primers for MALT1 in 1ul

and the 10mM dNTP mix of 1ul will be added.

- They'll then be heated at 65C for 3mins and then put on ice.
- Next, 4ul of the 5x Reverse Transcription (RT) buffer along with the 2ul of 100nM DTT, I ul of RNAase inhibitor and Iul of the RT enzyme mix will be added.
- The solution will be vortexed, centrifuged and then the supernatet will be collected for initial incubation of 45 mins at 42C and then 5min of 80C.
- qPCR will then be carried out using the specific MALT1 primers for one hour using the qPCR system from thermofisher.

Potential Alternatives: OCI-ly3 which is another ABC DLBCL cell line could essentially also be utilized because it also constitutes mutation in MALT1. However, HBL1 is employed a lot more because of having much more muatations such as in CARD11, BCL10 and CD79. Thus, it is much beneficial to study the mechanisms under a highly mutated environment.

Contribution to Research: This research will provide a novel insight to a feedback loop that perhaps be a cause of resistance. This research will also add great insights to the mechainms of a highly diverse pathway, which is still constantly being explored.

Risk and Safety: Student will work diligently to provide any harms to self as well as any damage to the equipment. Furthermore, fire safety training thorugh both online modules of the institution as well the physical training will be completed.

Quantitative analysis

Protein level: The western blotting results will be analyzed using the image lab software 6.0.1. Graphs will be fabricated based on the comparisons of the blot intensity to those of the loading control – actin.

Gene expression: Data from qPCR will be analyzed using the "relative method" where the CT values will be compared to different time points as well as treatments.

Bibliography:

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8. Fontan, Lorena et al. “Specific covalent inhibition of MALT1 paracaspase suppresses B cell lymphoma growth.” *The journal of investigation (2018)* 43974412. *PMC. Web. 02 Oct. 2018.*