Identify New Regulators of TNFR1-induced Necroptotic Pathway

VISITING MASTERS STUDENT
THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH
DEPARTMENT OF MEDICAL BIOLOGY, FACULTY OF MEDICINE,
DENTISTRY AND HEALTH SCIENCES
THE UNIVERSITY OF MELBOURNE
TSINGHUA UNIVERSITY

SUPERVISORS:
DR. GABRIELA BRUMATTI & PROF. JOHN SILKE

SUBMITTED IN TOTAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF MASTER OF RESEARCH
Table of Contents

List of Tables and Figures ................................................................. 3
Acknowledgements ............................................................................ 5
List of abbreviation........................................................................... 6
Abstract............................................................................................... 7
1. Introduction ..................................................................................... 8
   1.1 Leukaemia .................................................................................. 8
       1.1.1 Acute Myeloid Leukaemia .................................................. 8
   1.2 Cell Death Signalling ................................................................ 9
       1.2.1 TNF-receptor 1 Induced Cell Death .................................... 10
       1.2.2 Inhibitor of Apoptosis Proteins .......................................... 11
   1.3 Inhibitor of IAPs ...................................................................... 12
       1.3.1 Second mitochondria-derived activator of caspases (SMAC) .. 12
       1.3.2 SMAC-mimetic .................................................................. 12
       1.3.3 SMAC-Mimetics induced cell death ................................... 13
       1.3.4 Necroptosis and cancer ...................................................... 14
   1.4. Aims and Hypothesis ............................................................... 14
       1.4.1 Understand why AML cells are resistant to necroptosis ........ 15
       1.4.2 Identify new regulators of TNFR1-induced necroptotic pathway by CRISPR/Cas9 knock out screen ......................... 15
2. Material and Methods ................................................................. 16
   2.1 Cell Culture .............................................................................. 16
   2.2 Cell Death Assay .................................................................. 16
   2.3 Clonogenic Assay .................................................................... 16
   2.4 Transfection and Infection ....................................................... 17
   2.5 Western Blot .......................................................................... 17
   2.6 DNA Gel and Band Purification .............................................. 18
   2.7 DNA Sequence ....................................................................... 18
   2.8 gRNA preparation ................................................................... 19
   2.9 Transformation ........................................................................ 20
   2.10 CRISPR/Cas9 system ............................................................. 20
RESULTS ......................................................................................... 22
3. Factors of necroptosis resistance in KG-1 human AML cell line. .......... 22
   3.1 KG-1 is a human AML cell line which is resistant to TNFR1 induced necroptosis ......................................................... 22
   3.2 KG-1’s resistance is not caused by the lack of endogenous TNF production upon necroptotic stimuli .................................. 23
   3.3 KG-1’s necroptotic resistance is not caused by the loss of endogenous necroptosis-related proteins .................................. 24
3.4 Successful overexpression of wild type RIPK3 but not MLKL sensitises KG-1 cells to TNF induced necroptosis. .................................................................................................................. 25

3.5 Sequence results showed several mutant base pairs that may lead to the loss of function of KG-1 endogenous RIPK3 .................................................................................................................. 28

4. Using the CRISPR/Cas9 knock out system to identify new regulators of necroptosis in MV4;11 human AML cell line .............................................................................................................. 30

4.1. MV4; 11 is a human AML cell line which is sensitive to TNF induces apoptosis and necroptosis ................................................................................................................................. 30

4.2. Cas9 and gRNA expression will not make MV4;11 cells resistant to necroptosis ................................................. 31

4.3. Clonogenic assay selected clones which are resistant to necroptosis .................................................................. 32

4.4. Cell death assay confirmed most of the clones picked up from the clonogenic assay were resistant to necroptosis. ................................................................................................................ 33

4.5. These clones’ necroptotic resistances may be caused by the knockout of RIPK3 or MLKL .......... 34

4.6. gRNA sequence showed several attractive targets that may cause necroptotic resistance .......... 35

4.7 Validation of MAGEB3 ........................................................................................................................................ 37

4.7.1. MAGE family and MAGEB3 ............................................................................................................................. 37

4.7.2. Knock out MAGEB3 with either GeCKO or self-designed MAGEB3 gRNA will lead to necroptotic resistance in MV4;11 cell line ............................................................................. 39

4.7.3. Knock out MAGEB3 with either gRNA will downregulate the RIPK3 level ................................................. 41

4.7.4. Knock out MAGEB3 does not influence MAGEB3 mRNA level .................................................................. 42

4.7.5. Western blot could not detect the MAGEB3 protein level difference between parental cell line and MAGEB3 knock out clones ............................................................................ 43

4.7.6. cDNA sequence confirmed the MAGEB3 knockout ...................................................................................... 45

4.7.7. Knock out MAGEB3 in another human AML cell line, U937, cannot lead to necroptotic resistance .......................................................................................................................... 46

4.8 Validation of VWA7 ............................................................................................................................................. 48

4.8.1. Von Willebrand factor A domain containing 7 (VWA7) ................................................................................ 48

4.8.2. the result of knock out VWA7 in MV4;11 cells did not show necroptotic resistance ............................. 49

5. Discussion ......................................................................................................................................................... 51

5.1. Overexpression of necroptotic related proteins in KG-1 human AML cells .......................................................... 51

5.1.1. cDNA sequence detects several mutant base pairs which may lead to the loss of function of RIPK3 in KG-1 cells ............................................................................................................ 52

5.1.2. The limitations of this study and further improvement ................................................................................... 53

5.2. Identifying new regulators of necroptosis in AML ............................................................................................. 54

5.2.1. The mechanism of MAGEB3 and necroptotic resistance ............................................................................. 54

5.2.2. MAGEB3 antibody is defective ..................................................................................................................... 55

5.2.3. Knock out MAGEB3 in U937 cells does not lead to necroptotic resistance ................................................. 56

5.3. Other targets may lead to necroptotic resistance ............................................................................................... 57

5.3.1 VWA7 may not be a regulator of necroptotic pathway .................................................................................. 57

5.3.2. Some other targets which may lead to necroptotic resistance ................................................................... 57

6 Conclusion ......................................................................................................................................................... 59

7. References ......................................................................................................................................................... 60

Appendix .............................................................................................................................................................. 69

Supplemental figures ............................................................................................................................................... 69

Materials and Recipes ......................................................................................................................................... 72
List of Tables and Figures

Figure 1.1 TNF signalling pathway ................................................................. 11

Figure 3.1: KG-1 cells are responsive to birinapant mediated apoptosis but resistant to
TNF-induced necroptosis. .................................................................................. 24

Figure 3.2 Endogenous expression of necroptosis related proteins in AML cell lines. . 25

Figure 3.3 Successfully overexpression of RIPK3 in KG-1 cells ......................... 26

Figure 3.4 Overexpression of RIPK3 sensitises RIPK3 to necroptosis ................. 27

Figure 3.5. Sequence result of endogenous RIPK3 from KG1 cells. ................. 30

Figure 4.1 MV4;11 cells are responsive to birinapant mediated apoptosis and
necroptosis ........................................................................................................... 31

Figure 4.2 Cas9 and gDNA will not make MV4;11 cells resistance to necroptosis ..... 32

Figure 4.3 Most clones selected by clonogenic assay are resistant to necroptosis...... 34

Figure 4.4 Western blots for checking RIPK3 and MLKL level of MV4;11 clones ..... 35

Table 4.1. Combination of gDNA sequencing, Western Blot and cell death test ........ 36

Figure 4.5 Brief introduction of the MAGE protein family and MAGEB3 ............. 39

Figure 4.6 MAGEB3 knock out clones with either gDNA are resistant to necroptosis. 41

Figure 4.7 Time-coursing Western blot showed downregulation of RIPK3 in MAGEB3
knockout clones ................................................................................................. 42

Figure 4.8 DNA gel showed no difference of mRNA level between MV4;11 parental
cells and CRISPR knock out clones ..................................................................... 43

Figure 4.9 Endogenous expression of MAGEB3 and necroptosis related proteins in
AML cell lines. .................................................................................................. 44
Figure 4.10 Sequence results showed that the clones were MAGEB3 knock out clones
......................................................................................................................... 45

Figure 4.11 Cell death assay and Western blots showed that knock out MAGEB3 in
U937 was not related to necroptotic resistance ......................................................... 48

Figure 4.12 Cell death assay showed that knock out VWA7 in MV4;11 was not related
to necroptotic resistance .......................................................................................... 50

Figure S1. Sequence result of endogenous RIPK3 from MV4;11 cells...................... 69

Figure S2. MV4;11 clones may die by necroptosis with extracellular TNF .............. 71
Acknowledgements

I would like to begin by thanking my supervisor, Dr. Gabriela Brumatti and Prof. John Silke for allowing me to undertake this project. Your sustained support through meetings and experiments was a massive help to me. Thank you, Gabi, for teaching me all the experiment techniques and helping me whenever I needed, especially for improving my presentation and writing.

And a massive thank you to the Silke Lab and the rest of Inflammation Division for creating such a nice and amazing workplace that there were always many people willing to help me out with anything I had trouble with. Especially Natasha Silke, I think without Natasha I might not be able to run any base experiments and she was always there to solve my technique issues and answer my questions. Lin Liu, Oscar Zikou Liu and Daniel Frank also helped me a lot with my experiments, and they are all masters on Western blot who gave me a lot of suggestions on Western blot. Thank you, the rest of the lab, for helping me to finish my exchange Master study.

Thank you to WEHI FACS lab and Genomics lab for the use of your facilities to attain the key results that make up my thesis.

I would also like to thank my Master committee to attend all my confirmation seminars, give me suggestions and help me improve my presentation skills.

Thank you to the rest of the 2017 Tsinghua University exchange student for listening to my complaints and help me out of the dilemma I was facing.

And I extend my gratitude as a recipient of the Chinese Scholarship Community, which has been a tremendous help throughout the two years.

Finally, I would like to than my family for everything that they have done for me. These two years would not be possible without all of you.
List of abbreviation
AML  Acute Myeloid Leukaemia
ALL  Acute lymphoblastic leukemia
CML  Chronic myeloid leukemia
CLL  Chronic lymphocytic leukemia
RIPK  Receptor interacting protein kinase
TNF  Tumour necrosis factor
TNFR1  Tumour necrosis factor receptor 1
TRADD  TNFR-Associated Death Domain
TRAF2  TNF-Associated Factor 2
CIAP  Cellular Inhibitors of Apoptosis Protein
IKK  IκB kinase
NIK  NF-κB-inducing kinase
IKKα  IκB kinase-α
FADD  Fas-Associated protein with Death Domain
MLKL  Mixed lineage kinase-like
XIAP  X-linked IAP
SMAC  Second Mitochondria-derived Activator of Caspases
DLC  Dose Limiting Toxicity
DMSO  Dimethyl Sulfoxide
PI  Propidium iodide
TA  Acetate-EDTA
EB  Ethidium bromide
AGRF  Australian Genome Research Facility (AGRF)
Tari  Tariquidar
IDN  Emricasan
MDR1  Multidrug resistance 1 drug pumps
RHIM  RIP homotypic interaction motif
SHPK  Sedoheptulokinase
VWA7  von Willebrand factor A domain containing 7
MAGE  Melanoma Antigen Gene
MHD  MAGE homology domain
Pcgf3  Polycomb group ring finger 3
Abstract

Acute Myeloid Leukaemia (AML) is a vastly heterogeneous blood disorder with a poor prognosis for patients older than 65. Our group has been focused on developing new treatments for AML to replace the standard intensive chemotherapy. Previous data showed that the SMAC-mimetic birinapant in combination with the caspase inhibitor IDN could kill different types of AML both in vitro and in vivo through activation of necroptosis cell death pathway. However, over 50% of the patient samples tested in study showed resistance to necroptosis. This project aims to determine the molecular mechanisms that mediate necroptosis resistance in AML and identify new regulators of necroptotic pathway. The results obtained in this study will expand the knowledge of necroptosis signalling in leukaemia and will contribute to the optimal clinical use of birinapant/IDN drug combination. This project contains 2 parts; (1) We will use human AML cell lines that are resistant to necroptosis to determine the molecular changes involved in cell death resistance. (2) We will use CRISPR/Cas9 knock out screen in human AML cell lines that are sensitive to necroptosis, trying to identify new regulators of TNFR1-induced necroptotic pathway. Together these experimental approaches will allow a better understanding of the regulation of TNF-necroptosis signalling in AML.

By overexpression wild-type RIPK3 in the KG-1 cell line, we successfully sensitised KG-1 cells to necroptosis, which indicates that the KG-1 endogenous RIPK3 is dysfunctional. By cDNA sequence of KG-1 endogenous RIPK3, we detected several mutant base pairs, which may lead to the dysfunction, but this result needs further prove by genome sequence, which is undergoing.

By CRISPR knock out screen, we found several targets that may lead to the necroptotic resistant, and MAGE3 is the most research-worthy one. Knockout MAGEB3 in the MV4;11 cell line led to the downregulation of RIPK3 and the necroptotic resistance. However, this result could not be repeated on the U937 cell line, and the mechanism of how MAGEB3 regulates RIPK3 is still unclear. Further research will be done on MAGEB3 to have a better understanding of the role of MAGEB3 in the necroptotic pathway.

Together, all these results gave a better understanding of the necroptotic pathway and may contribute to the treatment of AML.
1. Introduction

1.1 Leukaemia

Leukaemia is a type of blood cancer which is a life-threatening malignant disorders of the blood and bone marrow[1]. The symptoms of leukemia may include bleeding and bruising, feeling exhausted, fever and an increased risk of infections[1, 2], which may be caused by the lack of normal blood cells. Diagnosis of leukemia is typically blood test or bone marrow biopsy. The exact cause of leukemia is unknown[3]. A combination of genetic factors and environmental factors are believed to play a role in leukemia-genesis, such as smoking, ionizing radiation, some chemicals (such as benzene), and Down syndrome[3, 4]. And people with a family history of leukemia also have a high risk of getting leukemia. There are four main types of leukemia: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML)[2, 5, 6]. Treatment of leukemia may involve some combination of chemotherapy, radiation therapy, target therapy, and bone marrow transplant, in addition to supportive care and palliative care as needed[2, 5]. The success treatment of leukemia depends on the age of the patient and the type of leukemia and outcomes have improved in the developed world[7]. The average five-year survival rate of all type of leukemia is 57% in the United States, and in children who are under 15 years old, the five-year survival rate is almost from 60 to 90%, depending on the type of leukemia[8, 9]. In 2015, leukemia was occurred in 2.3 million people and caused 353,500 death[2], and it is the most common type of cancer in children with the most leukemic cases being the acute lymphoblastic leukemia. However, in adults, the most common type appears to be CLL and AML[2].

1.1.1 Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is defined as a highly heterogeneous malignancy which results in the accumulation of immature myeloid progenitors, called “blasts”, in the bone marrow and peripheral[4, 10, 11]. The accumulation of blasts interferes with the homeostatic production of other differentiated myeloid cells, such as erythrocytes, granulocytes and monocytes [4]. Even though new treatments have been used to cure AML, only 40-45% of adult patients under 60 can achieve initial remission and over 50% of those eventually relapse. Thus, AML is still characterised by a low 5-year survival rate (approximately 25% Based on statistics from seer.cancer.gov) and a high relapse rate[4].
Apoptosis, a programmed cell death, is one of the critical mechanisms that influences the survival of AML cells, and resistance of apoptosis can possibly lead to the chemotherapy resistance and disease relapse[12-14]. As resistance to apoptosis is a common feature of AML, alternative cell death pathways have been explored in the attempt to overcome treatment resistance and disease relapse. Necroptosis, a relatively new type of cell death has been one of the cell death pathways exploited for the treatment of AML. Dr. Brumatti and colleagues have shown that induction of necroptosis by chemical compounds efficiently kill AML cells and prolong survival of in vivo models of the disease[15]. Moreover, necroptosis related proteins such as the receptor interacting protein kinase 1 and 3 (RIPK1 and RIPK3) have also been associated with the development of AML. Deficiency of RIPK1 and RIPK3 has been found in AML patients and it has been associated with the development of a more aggressive form of AML [16-18]. Together these studies suggest that both apoptosis and necroptosis have important role in the development and treatment of AML.

1.2 Cell Death Signalling

Cell death may be caused by several reasons; natural process of new cells replacing old dying cells; may result from factors such as disease, localised injury, or the death of the organism of which the cells are part. Cell death can occur in an uncontrolled manner or upon engagement of a molecular controlled pathway. Among the programmed cell death types, apoptosis pyroptosis, necroptosis and autophagy have had special attention in cancer research [15, 19]. These cell death pathways can be activated through a variety of mechanisms. For example, apoptosis, a programmed cell death extensively studied in the development and treatment of different cancers, can be activated by a receptor binding mechanism such as the tumour necrosis factor receptor 1 (TNFR1) (extrinsic pathway), or through the release of mitochondria-specific proteins (intrinsic pathway). But independent of the pathway activated, apoptosis is always characterised by the activation of cysteine-aspartic proteases (caspases). Active caspases induce degradation of specific cellular components leading to a cell death in with minimal effect on surrounding tissues. Caspases have also shown to play important role in other types of programmed cell death such as pyroptosis and necroptosis. Necroptosis, as a newly programmed caspase independent form of cell death, also play a critical role in cancer progression and has been regard as a clinical therapy to kill cancer cells with chemoresistance and apoptotic
resistance[20]. These forms of cell death are crucial to protect organism from stress signals and pathogenic attack.

1.2.1 TNF-receptor 1 Induced Cell Death
Tumour necrosis factor (TNF) signalling pathway has been implicated in both apoptosis and necroptosis cell death. Our group has focused on using TNFR1 induced apoptosis or necroptosis to initiating cell death to kill cancer cells, especially AML, for years. So, a better understanding of TNF signalling pathway contribute to a better clinical treatment of AML. Binding of TNF ligand to TNF receptor1 (TNFR1) (Figure 1.1), leads to a conformational change and formation of a membrane associated complex consisted of TNFR-Associated Death Domain (TRADD), TNF-Associated Factor 2 (TRAF2), Receptor Interacting Protein Kinase 1 (RIPK1) and cellular Inhibitors of Apoptosis Protein 1 or 2 (cIAP1/2)[21-23]. Within this complex, cIAP1/2 will promote the K63-linked polyubiquitylation of RIPK1[24, 25]. Polyubiquitylated RIPK1 promotes the recruitment of the IκB kinase (IKK) complex, leading to the downstream activation of canonical NF-κB pathway[24, 26, 27]. In resting cells, this caused the NF-κB translocation and transcription of its target genes which promote cell survival and proliferation[28, 29]. In the absence of cIAP1/2, the deubiquitated RIPK1 can form different complexes dependent on the activation of caspases and it will alternatively activate non-canonical NF-κB pathway. The central signalling fragment of non-canonical NF-κB pathway is NF-κB-inducing kinase (NIK), which will merge the TNF receptor signal and activate a downstream signal kinases, IκB kinase-α (IKKα), triggering p100 phosphorylation and processing[30, 31]. In some subtypes of cancer, the non-canonical NF-κB pathway will also induce the production of TNF to form a positive feedback loop. In the presence of caspases, deubiquitinated RIPK1 forms a complex IIA with TRADD, a procaspase-8 homodimer, a procaspase-8 heterodimer and Fas-Associated protein with Death Domain (FADD)[32-34]. The formation of complex IIA causes the autocatalytic cleavage and activation of procaspase-8, releasing the caspase-8 which will cleave and activate downstream caspases, such as caspase-3, leading to apoptosis[32, 34, 35]. If caspases are inactivated by endogenous inhibitors such as c-FLIP or chemical compounds such as ZVAD-fmk and IDN-6556, the deubiquituated RIPK1 forms a complex IIB with receptor interacting protein kinase 3 (RIPK3) and mixed lineage kinase-like (MLKL) protein, which will lead to autoactivation of RIPK3, causing the phosphorylation,
oligomerisation and translocation of MLKL to the cell membrane leading to disruption of the plasma membrane, release of intracellular contents [36] and necroptosis cell death.[37-40].

Figure 1.1 TNF signalling pathway

Different outcomes of TNF binding to TNFR1 based on the presence of IAPs and caspases. In the presence of cIAPs, ubiquitated RIPK1 leads to the activation of IKK complex and canonical NF-κB pathway. In the absence of cIAPs and present of activate caspases, the un-ubiquitinated RIPK1 will form complex IIa to initiate apoptosis. In the event of caspase inhibition, complex IIb, necrosome will form, leading to the activation of necroptosis. Figure elements provided by John Silke.

1.2.2 Inhibitor of Apoptosis Proteins

In mammals, the IAP family contains eight members, characterised by the presence of one to three Baculoviral (BIR) domains [41-43]. IAPs have many different functions; control of ubiquitin-dependent signalling events, caspase inhibition, and regulating the activation of NF-κB pathway [25, 44, 45]. Three of these mammalian IAPs; cIAP1, cIAP2 and X-linked IAP (XIAP) are involved in regulating both intrinsic and extrinsic apoptotic pathway by blocking the activity of caspases either directly or indirectly[46]. cIAPs can promote the ubiquitylation of RIPK1[25] and determine the outcome of TNF-receptor1 signalling, as mentioned before. cIAPs are also positive regulators of canonical NF-κB pathway and negative regulator of non-canonical NF-κB pathway [44, 47-49]. As
inhibitors of apoptosis cIAPs can indirectly inhibit activation of caspases via ubiquitation of Second Mitochondria-derived Activator of Caspases (SMAC). cIAPs promote the degradation of SMAC, releasing XIAP to bind and inhibit activation of caspase-3, -7 and -9 [45, 50-52]. Due to IAPs’ effectiveness in suppressing apoptosis, overexpression of these proteins lead to resistance to physiological cell death, which is an important hallmark of cancer[53, 54]. Acute Myeloid Leukaemia (AML) is one type of many cancers where IAP overexpression has been linked to chemoresistance and poor prognosis [55, 56].

1.3 Inhibitor of IAPs

1.3.1 Second mitochondria-derived activator of caspases (SMAC)
Inhibitor of apoptosis protein (IAPs) can be antagonized endogenously by SMAC/DIABLO [50, 57]. At the N-terminal, SMAC maintains a 55-residue mitochondria-targeting sequence, which is proteolytically removed when SMAC leaves the mitochondria by apoptotic stimulation[50]. This proteolytic process exposes SMAC’s Ala-Val-Pro-Ile (AVPI) tripeptide motif, which binds to XIAP, cIAP1 and cIAP2[58]. The cleaved version of SMAC can dimerise binding to BIR2 and BIR3 domains of XIAP, interfering with its direct effect on caspases[50, 59]. However, binding of SMAC to XIAP does not lead to the ubiquitylation and degradation of XIAP[60]. The AVPI motif can also bind to the BIR3 domain of cIAP1 and cIAP2. When bound, SMAC promotes the ubiquitylation and degradation of cIAP1 and cIAP2[60] allowing cell death to occur. Based on the mechanism of action of SMAC, small molecule drugs called SMAC-mimetics, were designed to mimic the endogenous activity of SMAC, inducing inactivation and degradation of IAPs.

1.3.2 SMAC-mimetics
The design of SMAC-mimetics compounds was based on the binding of endogenous SMAC’s AVPI motif to the BIR domains of IAPs. Many monovalent SMAC-mimetics were designed to possess a single BIR binding site[61, 62]. The binding of SMAC mimetics to IAPs allow degradation and inhibition of IAPs activity. It was then discovered that the endogenous SMAC can dimerise and bind to the BIR2 and BIR3 domains of XIAP simultaneously[63]. This prevents the BIR2 of XIAP binding and inhibition to caspase-3/-7, and BIR3 to caspase-9, releasing caspases from XIAP. Based
on this discover, bivalent SMAC-mimetics were designed to mimetic the interaction of dimeric SMAC[64]. Bivalent SMAC-mimetics have two identical AVPI mimetics, bound together with a linker[65]. Birinapant, one of the most outstanding bivalent SMAC-mimetics, has been in phase I/II clinical trials as a single compound treatment. Although no Dose Limiting Toxicity (DLC) was observed and cIAP1 was confirmed to be reduced in peripheral blood mononuclear and two colon cancer patients showed evidence of tumour regression, the efficiency of birinapant as a single therapy has been limited. A study conducted by Amaravadi and colleagues[66], has combined birinapant with chemotherapies such as paclitaxel/carboplatin, docetaxel, irinotecan, liposomal doxorubicin or gemcitabine. Compared to the outcome of the first phase I trial, 8 patients were reported with reversible Bell’s Palsy at higher doses of birinapant as a single agent, or combined with irinotecan, docetaxel or liposomal doxorubicin[67]. Together these results suggest that further optimisation of birinapant treatment is needed.

1.3.3 SMAC-Mimetics induced cell death
SMAC-mimetics act similarly to the endogenous SMAC via binding to IAPs, leading them to degrade.[68, 69]. When SMAC-mimetics such as birinapant binds to cIAP1/2, they induce self-ubiquitylation and degradation of cIAP1/2 leading to RIPK3 deubiquitylation and formation of complex IIa with FADD and procaspase-8[33, 60]. Auto-cleavage and activation of initiator caspase-8 in this complex leads to the activation of executioner caspase-3/-7 allowing apoptosis to occur [68]. Importantly, research developed in our group and others have also shown that, in contrast to conventional anti-cancer drugs, birinapant can also kill cancer cells by induction of necroptosis [15, 19]. The combined treatment of birinapant and caspase inhibitors result in the deubiquit of RIPK1 forming the complex IIb (necosome) with RIPK3 and MLKL, leading to the phosphorylation, oligomerization and translocation of MLKL to initiate necroptosis[15, 70-72]. The degradation of cIAPs by SMAC-mimetics also induces the production of TNF through RIPK1 and NF-κB pathway activation[73, 74], which leads to the activation of TNFR1[25], leading to a positive TNF-mediated feedback loop, eliminating cells through TNF-mediated apoptosis or necroptosis[73, 75]. Thus, Smac-mimetic have a dual mechanism of action offering an alternative therapeutic strategy for hard-to-treat cancers.
Although necroptosis has shown potential in the treatment of AML, findings from our laboratory revealed that 50% of the patient samples tested show resistance to birinapant induced necroptosis. Further understanding of the mechanisms of resistance of AML to necroptosis is crucial for advancing targeting of necroptosis into the clinic.

1.3.4 Necroptosis and cancer
Cancers, especially AML, are easy to build the resistance of apoptosis, leading to the resistance of chemotherapy[76-78], and necroptosis, as a newly identified programmed form of cell death, may be a novel approach to cure cancer, and several studies showed that using different drug combination treatment to induce necroptosis would kill cancer cells with apoptotic resistance or chemotherapy resistance[15, 19, 79]. Despite that necroptosis might be a novel way to cure cancers, necroptosis actually plays both a positive and negative role in cancer progression, like a double-edged sword[20, 80]. On the one hand, necroptosis plays a pro-tumour effect[81] because necroptosis will release intracellular components, such as DAMPs, which will promote inflammation, and lead to the activation of some signalling pathways, like NF-κB and MAPK pathways. The activation of these pathways has pro-tumour effects. On the other hand, necroptosis has an anti-tumour effect[82, 83] because necroptosis will release a strong immune signal that will interfere with tumour progression. And studies show that the role of necroptosis depends on the type and stage of cancers. So, a further understanding of necroptotic pathway will contribute to a better definition of relationship between necroptosis and cancer and the clinical utility of necroptosis.

1.4. Aims and Hypothesis
Silke laboratory has been focused in exploring necroptosis for the treatment of AML. Based on previous published work from Brumatti and colleagues, the combination of SMAC-mimetic and caspase inhibitor efficiently kills leukaemic cells in vitro and significantly prolongs the survival of murine models of AML, suggesting a new potential therapeutic strategy for AML [15, 19]. However, the same study has also shown that some AML patients are resistant to SMAC-mimetic and caspase inhibitor induced necroptosis. Thus, to further understand the molecular mechanisms by which AML cells develop
resistance to necroptosis, my project can be divided into two parts and I will mainly focus on TNFR1-induced necroptotic pathway.

1.4.1 Understand why AML cells are resistant to necroptosis.
Based on previous work developed in our laboratory, human AML cell line, KG-1, was shown to be sensitive to TNFR1-induced apoptosis but resistant to necroptosis. As previously described, TNFR1-induced apoptosis and necroptosis share the same upstream pathway. Ours and other published work have shown that despite resistance to necroptosis, KG-1 cells do express the effector proteins RIPK1, RIPK3 and MLKL involved [84] in the induction of necroptosis cell death. Thus, based on these observations this project focuses into two different hypotheses as why KG-1 cells are resistant to necroptosis:

(1) KG-1 cells may have mutations in RIPK3 or MLKL which may lead to the loss of function of these proteins.

(2) We hypothesise the existence of an intermediate inhibitor that would be implicated in the phosphorylation of MLKL, leading to inhibition of the necroptotic pathway.

By determining the mechanism of KG-1’s resistance to necroptosis we will be able to translate the findings to a clinical setting using patient samples, ultimately contributing to the optimal use of the combination of SMAC-mimetic and caspase inhibitor for the treatment of AML.

1.4.2 Identify new regulators of TNFR1-induced necroptotic pathway by CRISPR/Cas9 knock out screen
Published work from our laboratory show that MV4;11 leukaemic cell line is resistant to birinapant induced apoptosis but sensitive to necroptosis induced by the combination SMAC-mimetic plus caspase inhibitor. Thus, we utilise these cells to generate necroptosis resistant AML cells using an unbiased screen with CRISPR/Cas9 knockout technology. This experimental approach will allow us to define whether unknown regulators of TNFR1-induced necroptotic pathway are involved in AML necroptosis resistance.

Thus, this project is designed to increase our understanding of the mechanisms involved in the control of TNFR1-induced necroptosis. Ultimately the findings of this project will provide further insights of how necroptosis can be best targets in the clinic to improve the leukaemia patients’ outcomes.
2. Material and Methods

2.1 Cell Culture

A number of human and murine Acute Myeloid Leukaemia cell lines were used to achieve the aim of the project. Human Leukemic cell lines MV4-11, KG-1 and U937 were cultured in RPMI medium supplemented with 10% FCS. HEK 293T cells were cultured in DMEM medium supplemented with 10% FCS. Murine Leukemic cells were cultured in IMDM medium supplemented with 10% FCS and 3 ng/mL IL-3. All cell lines were cultured at 37°C in a 5% CO₂-humidified atmosphere.

2.2 Cell Death Assay

To determine the resistance to TNF induced necroptotic pathway or to address the therapeutic efficacy of different drugs as an MDR1i, cell death assays were performed. In a 96 well flat plate, 5 \times 10^4 cells were plated for each condition. All cell death assays included a Dimethyl Sulfoxide (DMSO) (vehicle) condition, plated at a concentration equal to the highest volume of the compound used in each test. Compounds were diluted with the cell culture media before plating to achieve the specific concentrations. Samples were incubated at 37°C (5% CO₂ injection). Human cells were treated for 48 hours, and murine cells were treated for 24 hours. Flow cytomet was performed at the specified time-points to distinguish live and dead cells based on analysis of size and propidium iodide (PI) uptake (1 μg/mL PI). Samples were acquired with FACS Calibur hardware. Live and dead cell populations were determined using FlowJo (version 10.1r5) software.

2.3 Clonogenic Assay

To select out cells truly resistant to necroptosis, clonogenic assays were performed. Clonogenic assay determines a given cell's clonogenic potential in chosen conditions. By incubating cells in a specified condition for approximately 14 days, cells which are resistant to the specified conditions will amplify and give clones. Clonogenic assays were set up by creating a RPMI media mixture containing 20% FCS and 0.3% agar. Media mixtures (without agar) were kept at 37°C within a water bath. 3% agar and drugs were added just before plating to avoid semi-solid media to set. Cells were counted and diluted in RPMI mix to a concentration of 5000 cells/mL. Mixtures were cooled for approximately 30 minutes without the lid to reach a semi-solid condition, then incubated
at 37°C for approximately 14 days. Clones resistant to SMAC-mimetics and caspase inhibitors were cultured in RPMI 10%FCS media for further analysis.

2.4 Transfection and Infection
To express the target protein or for CRISPR library screen, lentiviral transfection and infection were performed. For lentiviral transfection, 1×10^6 HEK 293T cells were plated in a 10 cm cell culture plate with 10mL DMEM (with 10% FCS) the day before transfection. DNA mix (2.5 μg plasmid of interest + 1.5 μg VSVG + 1 μg CMV), was diluted in 500 μL of EC buffer, containing 8 μL of enhancer per μg of DNA. Vortex and incubate the mixture at room temperature for 2-5 min. An equal volume (to enhancer) of effectene was added to the mixture and incubate it at room temperature for 5-10 min. During incubation, change media (10 mL DMEM with 8% FCS) for 293T cells. Add the mixture into the media equivalently and incubate the cells overnight. Change media (6 mL DMEM with 10% FCS) the following day and harvest virus after 24 hours.

For lentiviral transduction, 1×10^6 cells were resuspended in 1mL of the virus, spin in a 12-well cell culture plate with 4 μg/mL Polybrene at 2500 rpm for 90 mins and cultured with virus in a 5% CO₂ incubator for 16-18h.

2.5 Western Blot
Western blot was utilised to determine the relative amounts and/or the presence of specific proteins in specified conditions. Cell lysates were prepared as either a result of a time course treatment or CRISPR library screen result. Total cell lysates were prepared by lysing 1×10^7 cells/ml in SDS buffer (See Materials and Recipes) and boiling for 10 minutes. Samples were loaded into 26 well gel (NuPAFE Novex 4-12% Bis-Tris Nidi Protein Gels, Invitrogen) and ran at 80V for 10 minutes and 110V for 1.5 hours. Gel proteins were transferred to polyvinylidene fluoride or nitrocellulose membranes at 100V for 1 hour in Transfer Buffer (See Materials and Recipes). Membranes were blocked for 1 hour with Blocking Buffer (See Materials and Recipes), followed by incubation with an appropriately diluted primary antibody. Probing of membranes with primary antibodies was performed in cold room (4°C) for 16-20h. Membranes were then washed
3×10 minutes with Wash Buffer (See Materials and Recipes) and probed with specific secondary antibody. Membrane was incubated for 1 hour at room temperature, washed 3×10 minutes with Wash Buffer and coated with Immobilon Western Chemiluminescent Horse Radish Peroxidase Substrate. Protein expression was visualised via Chemidoc or Film and further analysed by ImageLab software. Membranes used for multiple probes were first washed in Wash Buffer before repeating the protocol.

2.6 DNA Gel and Band Purification
To check the result of PCR and get purified target DNA, DNA gel and band purification were performed.

For DNA gel, different concentrations of DNA gel were used (0.7%- 2%), depending on the size of the DNA to be analysed. The appropriate amount of agarose was dissolved in 1× Tris Acetate-EDTA (TA) buffer (See Materials and Recipes) with addition of 0.3 μg/mL of Ethidium bromide (EB). Gels were run at 120V for 40 minutes. DNA bands were visualised via Chemidoc and further analysed by ImageLab software. To purify DNA from a gel, band of interest was extracted and purified using Dark Reader (Clare Chemical Research) and Wizard SV Gel and PCR Clean-Up System. Measure the concentration of purified DNA with Nanodrop, and further use them for sequencing.

2.7 DNA Sequence
To detect if there is a mutation on the endogenous protein or check the CRISPR screen target, DNA sequence was performed. cDNA sequence and Genome sequencing were used throughout the project.

For cDNA sequence of KG1 cell lines, cDNA of the target gene was generated from a frozen pellet of 1×10⁷ cells. mRNA was extracted from cells using the standard protocol of RNeasy Kit (QIAGEN). For the generation of the cDNA library, Transcriptor First Strand cDNA Synthesis Kit (Roche) was used. Based on manufacture instructions, a Master mix containing 5 μL mRNA of target cells, 1 μL 50 pmol/μL Anchored-oligo(dT)$_{18}$ Primer, 4 μL 5× Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 μL 40 U/mL Protector RNase Inhibitor, 2 μL 10 mM each Deoxynucleotide Mix, 0.5 μL 20 U/μL Transcriptor Reverse Transcriptase and 7 μL of PCR-grade water was prepared and
incubated at 55°C for 30 minutes. Transcriptor Reverse Transcriptase was inactivated by heating to 85°C for 5 minutes and reaction stopped by incubating samples on ice. Samples were stored at -20°C for further experiments.

To enrich the target DNA, we used PCR to amplify the target gene. For a PCR reaction, a Master mix containing 1 × HF buffer, 0.3 mM/mL dNTPs, 1 μg/mL template, 2 mM/mL primer each and water to a final volume of interest. PCR reaction of samples was performed by the following steps: 1) initialization, 95°C for 1 minute; 2) denaturation, 95°C for 30 seconds; 3) annealing, temperature based on primers used in the experiments, for 30 seconds; 4) elongation, 72°C, time based on the target DNA fragment, usually 1 minute for 1000 base pairs; 34 repeats for step 3) and 4); 5) final elongation, 72°C for 5 minutes; 6) final hold, 12 °C for an indefinite time. To obtain a better sequencing result, purified PRC products were analysed by the Sanger Sequencing within the Australian Genome Research Facility (AGRF). The sequencing master mix consisted of 50 ng of PCR product, 10 pmol of sequencing primer and water (make the total volume to 12 μL). The sequencing results were analysed via SnapGene software.

2.8 gRNA preparation
To knock out genes with specific gRNA and avoid off-target effect, specific gRNAs were designed and cloned into plasmid with specific biomarkers.

gRNA were designed via website crispr.mit.edu or selected from CeCKO CRISPR V2 library, and ordered (sense and antisense strand) at IDT, with addition of tccc to the sense and aaac to the antisense strand at their 5-prime end. Before cloning the gRNAs into constitutive gRNA GFP vector, the vector was cut by BsmBI at 55 °C for an hour and purified. Annealed the sense and antisense strands in a 100 μL water-based culture with 16μM of each primer, 0.04 M NaCl, and 8 mM MgCl₂ with 65 °C heat for 5 minutes and cooled down for 1 hour.

For gRNA and digested plasmid ligation, a master mix was prepared with 4 μL of digested plasmid, 4 μL of aligned oligoes, 1 μL of ligation buffer and 1 μL of T-4. And kept the master mix at 16 °C overnight.
For gRNA clone confirmation, transform the ligation mixture the next day and picked clones for Miniprep (4 mL LB with 1 µg/mL Ampicillin). Digested the Miniprep with BamHI and BsmBI and picked up preps which showed a linearized digested products on DNA gel and sent them for sequence with specific primer, following the step mentioned before.

2.9 Transformation
To amplify plasmid, transformation and plasmid extraction were performed.

For transformation, competent bacteria and heat shock were used. Competent bacteria were thawed on ice and 50 µL of bacteria was dispensed into a pre-chilled 13 mL bacteria culture tube. Added 1 µL of plasmid and mixed well with pipette tip. Incubated the mixture on ice for 30 minutes and heat-shocked cells at 42 °C for 40 seconds and recovered cells on ice for 2 minutes. Then added 450 µL of LB and incubate the bacteria in a 37 °C shaker incubator for 1 hour and plated approximately 150 µL on Amp' plates and incubated at 37 °C overnight.

For plasmid amplification, depending on the amount of plasmid needed, different volumes of cultures were chosen. Incubate bacteria with appropriate volume of culture for approximately 12 hours and extracted the plasmid with plasmid kit (QIAGEN). Kept the plasmid at -20 °C for further use.

2.10 CRISPR/Cas 9 system
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a family of DNA sequences that are found within the genomes of prokaryotic organisms, such as bacteria and archaea[86]. These sequences are derived from DNA fragments from viruses which have previously invaded the prokaryote and are used to detect and destroy DNA from similar viruses during subsequent infections. So, these sequences play a crucial role in the antiviral defence system of prokaryotes[86].

CRISPR-associated protein 9 (Cas9) is an enzyme, which uses CRISPR sequences as a guide to detect and cleave specific strands of DNA that are complementary to the CRISPR sequence. Cas9 proteins, together with CRISPR sequences, form the basis of a technology
known as CRISPR-Cas9, which can be used to edit genes within organisms[87]. This editing process has a wide variety of applications, including the development of biotechnology products, basic biological research, and treatment of diseases[88]. For the CRISPR/Cas9 screen used in the experiments, a CRISPR library was used, which is Human CRISPR Knockout Pooled Library (GeCKO v2) prepared by Zhang lab[89]. This library has 6 sgRNAs per gene and 1000 control sgRNAs, which are designed not to target the genome. The library also have sgRNAs that target the miRNA. The library used in the experiments was in a 2-plasmid format. The sgRNA was in a lentiGuide-Puro vector, which has the advantage of the higher titer for the library virus, but required the cell used in the screen contain Cas to perform the knock out system.
RESULTS

3. Factors of necroptosis resistance in KG-1 human AML cell line.

3.1 KG-1 is a human AML cell line which is resistant to TNFR1 induced necroptosis

Preliminary data from our laboratory suggested that human AML cell line KG-1 is resistant to TNFR-1 induced necroptosis. To further validate these results, cell death assays with a titration of birinapant combined with Tariquidar (Tari), Emricasan (IDN), or TNF were performed on KG-1 cells and sorted into living and dead cell populations using flow cytometry. From the result, 200 nM or even 2 µM birinapant treatments were not able to kill these cells by SMAC-mimetic induced apoptosis (Figure 3.1). Concurrent studies from our laboratory have shown that the SMAC-mimetic birinapant, which is mostly used in this study, is a substrate of multidrug resistance 1 drug pumps (MDR1). KG1 cells have been shown to overexpress MDR1, thus suggesting they are able to pump out birinapant and are resistant to birinapant mediated apoptosis [90, 91]. To test this hypothesis, Tari, a MDR1 inhibitor[92], was used in combination with birinapant to overcome treatment resistance. The cell death results showed that 200 nM of birinapant, in combination with 1 µM of Tari, killed over 80-90% of KG-1 cell (Figure 3.1). When looking at the necroptotic induced cell death mediated by the co-treatment of birinapant and the caspase inhibitor IDN, addition of Tari up to 48 hours treatment, had no effect, with cell death rate being observed at approximately 15% (Figure 3.1), similar to the control group. Together, these results emphasise the result that KG-1 is a cell line which, in the presence of MDR1 inhibitor, is sensitive to TNFR1 induced apoptosis but resistant to TNFR1 induced necroptosis.

As mentioned before, TNFR-1 induced apoptosis and necroptosis share the same upstream pathway. Because KG-1 cells can be killed by birinapant induced apoptosis, these results indicate that TNFR1 upstream pathway such as receptor and ligand can function normally and thus defects downstream the receptor/ligand complex may be responsible for the necroptosis resistance observed.
3.2 KG-1’s resistance is not caused by the lack of endogenous TNF production upon necroptotic stimuli

To initiate the necroptosis, TNF is required. When using SMAC-mimetics to induce cell death, it will activate the non-canonical NF-κB pathway to produce TNF. Thus, we hypothesise that if KG-1 cells are not able to produce TNF upon birinapant and IDN treatment, which may cause resistance to necroptosis. To validate this hypothesis, KG-1 cells were co-treated with birinapant and IDN in the presence or not of exogenous TNF. Cell death was analysed by Propidium iodide incorporation via flow cytometry. According to results, even when cells were treated birinapant, IDN and 100 ng/mL TNF, KG-1 cells were resistant to this necroptotic stimuli with cell death rate lower than 20% (Figure 3.1), which was close to the control treatment. These results suggested that, upon birinapant treatment, KG-1 cells are able to produce TNF and thus the resistance to necroptosis is not related to the lack of self-production of TNF.
**Figure 3.1: KG-1 cells are responsive to birinapant mediated apoptosis but resistant to TNF-induced necroptosis.**

Cell death assays in KG-1 cells were performed using different drugs combinations. Cells were treated with birinapant (0, 0.2 and 2 µM) in the presence or not of 1 µM of Tari, 5 µM of IDN, and 100 ng/mL TNF. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM. Dot = a single data. Error bar = SEM.

### 3.3 KG-1’s necrototic resistance is not caused by the loss of endogenous necroptosis-related proteins

Previous results in the group and some other literature showed that KG-1 expresses the key necroptosis related proteins RIPK3 and MLKL[84]. Thus, suggesting that KG-1’s resistance to TNFR1-induced necroptosis is not associated with the loss of these proteins.

To validate these results and determine expression level of TNFR1 signalling related proteins such as RIPK1, RIPK3 and MLKL, western blots were performed on total cell lysates of KG-1 cells. MV4;11 is a human AML cell line which is sensitive to TNFR-1 induced necroptosis and has endogenous level of RIPK3 and MLKL[15], thus it was used here as a positive control. Western blots showed that KG-1 has endogenous RIPK3 (Figure 3.2). However, compared to MV4;11, expression appears reduced. This may be caused by variation on the total amount of protein loaded. Anti-actin loading control blots also showed lower expression in KG-1 cells when compared to MV4;11 (Figure 3.2).

When analysing the expression of MLKL and RIPK1, KG-1 cell presented equal levels of RIPK1 and MLKL, when compared to MV4;11 cells (Figure 3.2). Together these results indicated that KG-1 cells have endogenous RIPK3 and MLKL and should be able to respond to necroptotic stimuli. The lack of response to necroptosis stimuli suggests that loss of function mutations in RIPK3 or MLKL may be present in KG-1 AML. Alternatively, an intermediate inhibitor may also be implicated in the phosphorylation of MLKL, leading to inhibition of the necroptotic pathway.
3.4 Successful overexpression of wild type RIPK3 but not MLKL sensitises KG-1 cells to TNF induced necroptosis.

To determine whether KG-1 cells may have mutations in RIPK3 or MLKL which may lead to the loss of function of these proteins and thus resistance to necroptosis, human species wild type RIPK3 and MLKL were overexpressed in KG1 cells and sensitivity to necroptosis determined by cell death analysis (Figure 3.3 A). Overexpression of these proteins was induced by transduction of viral particles followed by puromycin selection and expansion after infection. To confirm overexpression of wild type RIPK3 and MLKL, protein expression was induced in KG-1 cells by doxycycline treatment and total cell lysates analysed by western blot. Our results showed a strong band for RIPK3 (Figure 3.3 B), validating the over expression system. In this same western blot, endogenous level of RIPK3 could not be detected. This may be caused by the strong overexpression signal since previous results have already showed that KG-1 cells have endogenous level of RIPK3 (Figure 3.2). When analysing MLKL expression, there was almost no difference
between KG-1 cell with wild type MLKL with or without doxycycline treatment (Figure 3.3B). This result suggests that these cells are not able to overexpress wild type MLKL.

**Figure 3.3 Successfully overexpression of RIPK3 in KG-1 cells**

(A) Schematic of the analysis of why KG-1 cells were resistant to necroptotic stimuli on the aspect of mutant endogenous proteins (RIPK3 or MLKL). (B) Western bot probing for RIPK3, MLKL and actin in MV4;11 cell line, KG-1 parental cell line (Ø) and KG-1 with wild type RIPK3 (RIPK3) or MLKL (MLKL) in the presence (+) or not (-) of 1 μg/mL doxycycline treatment for 16h.

To test whether overexpression of wild type RIPK3 would sensitisise KG-1 cells to necroptosis, cell death assays were performed. Cells were treated with 1 μL birinapant combined with 1 μL Tari or 10 μL IDN, in the presence or not of 1 μg/mL doxycycline and analysed by living and dead cell populations using flow cytometry. The cell death results of the parental cell line showed that these cells were still sensitive to apoptosis and resistant to necroptosis (Figure 3.4A), and doxycycline overnight treatment did not cause more cell death which suggested that doxycycline was not toxic to these cells (Figure 3.4B). The cell death result of KG-1 cells with wild type RIPK3 and without doxycycline
treatment showed that these cells were resistant to the co-treatment of birinapant and IDN (Figure 3.4A). The cells induced to express wild type RIPK had a high percentage of viability in the absence of treatment, suggesting that overexpression of wild type RIPK3 is not lethal to these cells (Figure 3.4B). When these same cells reconstituted with wild type RIPK3 and treated with doxycycline were treated with birinapant and IDN to induce necroptosis they were killed approximately 50% compared to the parental cells, the cell death rate was 10-20% (Figure 3.4B). This result showed that overexpression of wild type RIPK3 sensitises KG-1 cells to necroptosis. According to the previous results, we hypothesise that KG-1 cells have a mutant form of RIPK3 which leads to the resistance to necroptosis.

![Figure 3.4](image)

**Figure 3.4** Overexpression of RIPK3 sensitises RIPK3 to necroptosis

(A and B) Cell death assay on MV4;11, KG-1 and KG-1 reconstituted cells in the presence (+) or not of doxycycline (1 µg/mL) for 16h. n=3 independent experiments. Values represents means ± SEM. Dot = a single data. Error bar = SEM.
3.5 Sequence results showed several mutant base pairs that may lead to the loss of function of KG-1 endogenous RIPK3

To detect whether there are mutant base pairs on KG-1 endogenous RIPK3 mRNA, which will lead to the loss of function, Sanger sequencing was performed by the Australian Genome Research Facility (AGRF). Total mRNA was extracted from KG-1 cells, and cDNA of KG-1 endogenous RIPK3 was generated with specific primer and total mRNA via PCR (see Material and method).

According to the alignment of KG-1 endogenous RIPK3 sequence result and the RIPK3 cDNA profile, several mismatches base pairs which may lead to the loss of function were detected (Figure 3.5A). The first several mismatches were at the beginning of the sequence result and the others were at the ending of the sequence result. The part at the ending of the sequence result may not have a major influence on KG-1 endogenous RIPK3 function because this part of mismatches was already after the stop codon (for KG-1 endogenous RIPK3, the stop codon is TGA and the RIPK3 profile stop codon is TAA) which means these mismatch base pairs are not engaged in translation (Figure 3.5C). To avoid the error that may occur in the PCR and cDNA sequence, the RIPK3 cDNA of MV4;11 cells was also generated and sequenced in parallel as a positive control.

And the mismatch base pairs at the beginning may cause the loss of function of KG-1 endogenous RIPK3. When look at the red labelled ATG (Figure 3.5B), which is the start codon for RIPK3. KG-1 endogenous RIPK3 had a TCC instead of the ATG (Figure 3.5B), which means that the KG-1 endogenous missed this start codon and the next start codon is around 130 base pairs after the first start codon (Figure 3.5A). And the sequence result of MV4;11 endogenous RIPK3 showed a normal start codon, which indicated that this mismatches might not be caused by PCR or cDNA sequence (Figure S1). If KG-1 endogenous RIPK3 used the second start codon, it will lose approximately 35 amino acids at the N-terminal and eventually have a truncated form of RIPK3, but this truncated form of RIPK3 was not detected by the RIPK3 antibody on the previous Western blot (Figure 3.2). RIPK3 has two domains, at the N-terminal is the kinase domain and at the C-terminal is the RIP homotypic interaction motif (RHIM) domain. If the KG-1 endogenous RIPK3 lose several amino acids at the N-terminal, it may interfere with the normal function of
the kinase domain thus stopping the necroptotic signalling and making KG-1 cells resistant to necroptosis.

This mutant result supports the hypothesis of whether KG-1 cells may have mutations in RIPK3, which may lead to the loss of function of these proteins and cause the necroptotic resistance.
Figure 3.5. Sequence result of endogenous RIPK3 from KG1 cells.

(A) cDNA alignment of RIPK3. Comparison of RIPK3 from KG1 (upper sequence) and RIPK3 coding region (lower sequence, obtained from NCBI; NCBI Reference Sequence: NM_006871.4). The red arrows show the sequencing primers’ binding sets and orientation. Red labels highlight the mismatch base pairs. (B) Highlight the mismatches at the 3’ end. (C) Highlight the mismatches at the 5’ end.

4. Using the CRISPR/Cas9 knock out system to identify new regulators of necroptosis in MV4;11 human AML cell line

4.1. MV4; 11 is a human AML cell line which is sensitive to TNFR1 induced apoptosis and necroptosis

To validate the previous result obtained in the group that MV4;11 cells are sensitive to TNFR-1 induced necroptosis, cell death assays were performed on MV4;11 cells and analysed by living and dead cell populations using flow cytometry. From the results of the cell death assays, 200 nM or 2 µM of birinapant treatment was not able to kill these cells by apoptosis (Figure 4.1). This resistance may be caused by these cells are not able to product TNF under apoptotic stimuli. When extra TNF was added, MV4;11 cells dead by approximately 80% (Figure 4.1), which indicated that these cells are sensitive to apoptosis.

As shown in the results, co-treatment of 2 µM of birinapant and 10 µM of IDN killed these cells by 80-90% (Figure 4.1), which suggested that these cells are sensitive to TNFR1 induced necroptosis. Previous results of this thesis also showed that these cells have necroptotic essential downstream proteins (RIPK1, RIPK3 and MLKL). Together these results showed that MV4;11 is a suitable cell line for the CRISPR knock out screen to generate necroptotic resistant clones.
4.1 MV4;11 cells are responsive to birinapant mediated apoptosis and necroptosis

Cell death assays in KG-1 cells were performed using different drugs combinations. Cells were treated with birinapant (0, 0.2 and 2 µM) in the presence or not of 1 µM of Tari, 5 µM of IDN, and 100 ng/mL TNF. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM.

4.2. Cas9 and gRNA expression will not make MV4;11 cells resistant to necroptosis

To check if the expression of Cas9 or gRNA will influence the features of MV4;11 cells, cell death assay was performed on MV4;11 cells after these cells were infected with Cas9 vector, empty gRNA vector and both of these two vectors. Cells were first selected by the biomarker contained in the vector then treated with birinapant or birinapant with IDN. As shown in the results, Cas9, empty gRNA vector or both of the plasmids didn't make these cells resistant to necroptosis (Figure 4.2). These results suggested that the vectors and the cells are suitable materials to do the CRISPR knock out screen.
Figure 4.2 Cas9 and gRNA will not make MV4;11 cells resistant to necroptosis

Cell death assays in MV4;11 cells with/without Cas9 or gRNA plasmid were performed using different drugs combinations. Cells were treated with 1 µM of birinapant or 1 µM birinapant and 10 µM IDN. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=2 independent experiments. Values represent means ± SD.

4.3. Clonogenic assay selected clones which are resistant to necroptosis.

After MV4;11 cells were infected with Cas9 vector and human GeCKO v2 library, and selected for the puromycin resistance (which contained by GeCKO library), clonogenic assays were performed to select clones resistant to necroptotic stimuli. After approximately 14 days of treatment, a large number of clones appeared in the control plates or the plates containing only birinapant (caused by these cells were resistant to birinapant alone treatment). Only a few clones appeared in the plates containing both birinapant and IDN, which suggested that clonogenic assay can select clones resistant to the necroptotic stimuli.
4.4. Cell death assay confirmed most of the clones picked up from the clonogenic assay were resistant to necroptosis.

To confirm the clones picked up from clonogenic assays are truly resistant to necroptosis, instead of directly mutation analysis, cell death assays were performed on MV4;11 clones and sorted into living and dead cell populations using flow cytometry. The procedures were repeated twice, and 8 clones were picked from the first repeat and 12 clones were picked from the second.

The cell death result from the first repeat showed the cell death rates of clones 1, 2, 3, 5, and 8 were approximately 10% (Figure 4.3A) compared to the parental cells, which were 55% (Figure 4.3A). And the condition of the cells was good when observed with microscopes. This result showed that these clones are truly resistant to necroptosis. But the cell death percentage of clone 4, 6, and 7 were similar to the parental cells (Figure 4.3A), which showed that the setup of the clonogenic assay was not proper enough to select the resistant clone efficiently. This TNF dependent resistance may be caused by the half-life of IDN is short[93], and the duration of the clonogenic assay is quite long. And when these clones were treated with necroptotic stimuli and extra TNF, clone 2, 5, and 8 appeared to be sensitive to necroptosis, cell death rate change from 10% to 90% (Figure S2A).

This result may be caused by the knock out of a specific protein interferes with the self-production of TNF under necroptotic stimuli. So, in the next repeat, to avoid the comeback of the necroptotic clones, double concentration of IDN was used in the clonogenic assay. To avoid the TNF dependent necroptotic resistant clones, extra TNF was added in the clonogenic assay.

The cell death result from the second repeat showed the cell death rates of all the clones were approximately 10% compared to the 60% of the parental cells (Figure 4.3B). And the condition of all the clones was good when observed with microscopes. Moreover, when these clones were co-treated with necroptotic stimuli and extra TNF, the cell death rates remained the same, which suggested that all the clones obtained from the second repeat are genuinely resistant to necroptosis (Figure S2B).
Together, all these 20 clones obtained from the clonogenic assays would be further analysed to understand the mechanism of this necroptotic resistance.

Figure 4.3 Most clones selected by clonogenic assay are resistant to necroptosis.

Cell death assays in MV4;11 clones were performed using different drugs combinations. Cells were treated with birinapant (1 µM) in the presence or not of 10 µM of IDN. SI stands for Bir + IDN. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM.

4.5. These clones’ necroptotic resistances may be caused by the knockout of RIPK3 or MLKL

To check if the necroptotic resistance was caused by the knockout of vital downstream proteins (RIPK3 or MLKL), Western blots were performed to check out the protein levels.
in the necroptotic resistant clones. The Western blots for the first 8 clones showed that clone 1 might be an MLKL knock out clones (Figure 4.4), and it is widely known that knock out of MLKL will make cells resistant to necroptosis. The Western blot for the second 12 clones showed that all of these clones have MLKL (Figure 4.4), but the blot probing for RIPK3 showed that all of the clones, except clone 14, have no endogenous RIPK3 protein (Figure 4.4), and deficiency of RIPK3 will lead to necroptotic resistance was proved before.

All of the Western blots gave a negative result which indicates that all of my targets in the screen have been proven before. However, I got suggestions from my supervisors that my screen targets may not directly target RIPK3 or MLKL, instead, they may play an essential role in transcription, translation or post-translation modification of RIPK3 or MLKL.

![Figure 4.4 Western blots for checking RIPK3 and MLKL level of MV4;11 clones](image)

Figure 4.4 Western blots for checking RIPK3 and MLKL level of MV4;11 clones

Total cell lysates of MV4;11 parental cells and knock out clones were analysed for expression of proteins related to TNF mediated necroptosis. Probe of lysates with the indicated antibodies, RIPK3 and MLKL. Data representative of 2 independent experiments.

4.6. gRNA sequence showed several attractive targets that may cause necroptotic resistance

To validate the hypothesis that my genome screen was not directly targeting RIPK3 or MLKL, gRNA sequence was performed. The whole genome of all the 20 clones was extracted and sequenced by Genomics Laboratory, Advanced Technology & Biology, Walter & Eliza Hall Institute.
## Table 4.1. Combination of gRNA sequencing, Western Blot and cell death test

When combining the sequence result and, Western blot and cell death result together, several clones were picked out.
The gRNA of clone 1 was MLKL, and from Western blot, clone 1 was an MLKL negative clone, which showed that our CRISPR/Cas9 knock out worked and it was not an off-target effect.

Clone 2 and clone 4 both had the gRNA of sedoheptulokinase (SHPK), the Western blots showed that they both had RIPK and MLKL, and the cell death assay showed that they were TNF dependent necroptotic resistant.

Clone 14 showed to have the RIPK3 and MLKL protein, and it was resistant to necroptotic stimuli, which may be caused by this clone had loss of TNF receptor superfamily member 1A(TNFR1), which will completely stop the necroptotic pathway.

Clone 3 had the gRNA of von Willebrand factor A domain containing 7 (VWA7), this clone had an endogenous level of RIPK3 and MLKL, and this clone is resistant to birinapant, IDN and TNF treatment. This VWA7 may be a new regulator of the necroptotic pathway.

Clone 9, 11, 12, 16 and 20 all had the gRNA of Melanoma Antigen Gene 3 (MAGEB3), the Western blots showed that they all had MLKL but no RIPK3, and the cell death assay showed that they were resistant to birinapant and IDN treatment even with extra TNF. This result showed that 5 out of 20 clones had the gRNA of MAGEB3 and they had a deficiency in RIPK3, which indicated that knock out MAGEB3 in MV4;11 cell line leads to the downregulation of RIPK3 and the resistant of necroptosis.

From the sequence result, VWA7 and MAGEB3 were picked out for further research.

4.7 Validation of MAGEB3
4.7.1. MAGE family and MAGEB3
To validate the effect that knock out MAGEB3 will downregulate the endogenous RIPK3 level and cause the necroptotic resistance in MV4;11 cells, a better understanding of MAGE family and MAGEB3 are required.
The Melanoma Antigen Gene (MAGE) protein family is a highly conserved, large group of proteins which was discovered initially as an antigen on tumours. MAGE family proteins were used as immune-targets[94] because they were not expressed in most normal tissues except testis and re-expressed in cancers. The proteins in the MAGE family can be divided into two subfamilies, depending on their expression. Type I MAGE proteins contain the subfamily A, B and C, and these genes are located on chromosome X (Figure 4.5A)[95]. Type II MAGE proteins contain D, E, F, G and Necdin, and these genes are nor restricted on chromosome X (Figure 4.5A). All MAGE subfamilies contain a MAGE homology domain (MHD) which is around 170 amino acids (Figure 4.5B)[96]. Recently, studies showed that MAGE family proteins could bind to specific E3 ligase via their MHDs, thus adjusting the activity of the specific E3 ligase. And the adjustment can be achieved in several different ways, such as directly enhance the activity of the specific E3 ligase; changing the location of the specific E3 ligase; specifying the targets of the particular E3 ligase, or some no-ubiquitination function effect (Figure 4.5C)[96-98].

As for MAGEB3, not many researches have been focused on MAGEB3 and this protein used to be an immune-targets of cancers. Recently, studies showed that MAGEB3 is involved in X chromosome inactivation as a critical interactor with Polycomb group ring finger 3 (Pcgf3), which is ring finger protein binding onto the inactivated chromosome X [99]. And MAGEB 3 also showed an enrichment in nucleoli which also indicates that MAGEB3 may influence endogenous protein level on a transcription level[99].

When checking MAGEB3 mRNA level in different cancers, the results showed that MAGEB3 was downregulated in many different types of cancers except kidney cancer (Figure 4.5D). If downregulation of MAGEB3 relates to the development of necroptotic resistance, downregulation in many types of cancers may contribute to the development of cancers. Several studied have shown that the expression of key regulators of necroptotic pathway in cancer cells is generally downregulated, which suggest that cancer cells may also evade necroptosis to survive[20].
Figure 4.5 Brief introduction of the MAGE protein family and MAGEB3

(A) Phylogenetic tree of eukaryotic MAGEs. (B) A list of human MAGE protein family and their common MHD domain. (C) Illustration of mechanism of how MAGE proteins regulate the specific E3 ligase. (D) MAGEB3 mRNA level in different cancers, data from oncomx.org/searchview

4.7.2. Knock out MAGEB3 with either GeCKO or self-designed MAGEB3 gRNA will lead to necroptotic resistance in MV4;11 cell line.

To validate the result that knock out MAGEB3 will lead to necroptotic resistance in MV4;11 cell line and avoid the off-target effect, another gRNA for MAGEB3 was designed and used with GeCKO gRNA (see Material and Method) from the screen were used to knock out MAGEB3 in MV4;11 cells.

After infection and selection, to confirm whether knock out MAGEB3 will cause the necroptotic resistance, 4 clones for each gRNA were chosen by GFP positive contained by gRNA plasmid via flowcytometry and used for cell death assay.
From the result of GeCKO gRNA clones, all of these clones died by 10% with birinapant and IDN treatment, comparing to the parental cell line, the cell death rate was approximately 60% (Figure 4.6A), and the condition of the cells was good when observed with microscopes. Moreover, these cells still responded to apoptosis. All these results consistent with the results got from the CRISPR knock out screen.

From the result of self-designed gRNA clones, compared to parental cell line, 3 out of 4 clones did not respond to necroptotic stimuli and responded to apoptotic stimuli (Figure 4.6B), which confirmed the result of the CRISPR knock out screen. The clone which responded to necroptotic stimuli may be caused by the gRNA designed by myself was not as effective as the gRNA from the library or the gRNA was not expressed in the cells (because the cells were only selected for the bio marks).

All of the results from the cell death assay showed that knock out MAGEB3 in MV4;11 cells and lead to necroptotic resistance.
Figure 4.6 MAGEB3 knock out clones with either gRNA are resistant to necroptosis

Cell death assays in MV4;11 MAGEB3 knock out clones were performed using different drugs combinations. Cells were treated with 1 µM birinapant in the presence or not of 1 µM of Tari, 10 µM of IDN, and 20 ng/mL TNF. SI stands for Bir + IDN, TS stands for TNF + Bir, and TSI stands for TNF + Bir + IDN. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM.

4.7.3. Knock out MAGEB3 with either gRNA will downregulate the RIPK3 level

To validate the effect that knock out MGAEB3 will downregulate the endogenous RIPK3 level, which may be the reason of necroptotic resistance and check the bio markers for necroptosis (phosphor-MLKL), 1 clone for each gRNA was chosen and a time-coursing of Bir and IDN treatment Western blot was performed.

The Western blot probing for phosphor-MLKL in parental cell line showed a weak band when the time point reached 4 hours’ treatment, and a strong band appeared at 6 hours (Figure 4.7), which indicated that these cells would die by necroptosis. The Western blot for GeCKO gRNA clone and self-designed gRNA clones has no bands for phosphor-MLKL even the time point reached 6 hours (Figure 4.7), which indicated that these cells would not die by necroptosis. These results were consistent with the results of the cell death assay.

The Western blot probing for RIPK3 showed that both CRISPR MAGEB 3 knock out clones did not have the endogenous level of RIPK3 compared to the strong band of the parental cells (Figure 4.7), which confirmed the result obtained before that knock out MAGEB3 would downregulate the endogenous level of RIPK3, causing the necroptotic resistance.

The Western blot probing for MLKL showed that the CRISPR MAGEB3 knock out clones have the same MLKL level as the parental cells (Figure 4.7). This result indicated that the samples were loaded and knock out MAGEB3 may not influence other protein levels in MV4;11 cells.
All these results were consistent with the results obtained before, and further research was done on MAGEB3 to discover the relationship between MAGEB3 and RIPK3.

4.7.4. Knock out MAGEB3 does not influence MAGEB3 mRNA level

To understand the mechanism how MAGEB3 regulates the level of RIPK3, mRNA of the CRISPR screen clone 1, 3, 9, 11, 12, 13, 16, 18 and 20 were extracted and the cDNA of MAGEB3, RIPK3, MLKL and GAPDH were generated. DNA gel was performed to measure the cDNA level.

The DNA gel for MLKL showed that all the clones have the mRNA of MLKL (Figure 4.8), even though the clone 1 was supposed to be an MLKL knock out clone. This result indicated that knock out of a protein might not directly interfere with the mRNA level of this protein.
The DNA gel for MAGEB3 showed that MV4;11 cell line has endogenous mRNA of MAGEB3 (Figure 4.8). However, there was no difference between parental cell line and MAGEB3 knock out cells (Figure 4.8), which is possible because CRISPR knock out may not directly interfere with the endogenous mRNA level.

The DNA gel for RIPK3 showed that all the clones have mRNA of RIPK3 (Figure 4.8), even though different clones have different amounts of DNA, there is no significant difference between parental cell line and the MAGEB3 knock out clones. This result indicates that MAGE3 may not regulate RIPK3 level via transcription.

![DNA gel](image)

**Figure 4.8 DNA gel showed no difference of mRNA level between MV4;11 parental cells and CRISPR knock out clones**

Total cell mRNA was extracted from the CRISPR knock out screen clones, cDNA library was synthesized via cDNA kit (see Material and Method), and cDNA for each target gene was synthesized by PRC with specific primers and cDNA library. Samples were run in 1 % agarose gel and detected by Chemidoc. Data representative of 2 independent experiments.

**4.7.5. Western blot could not detect the MAGEB3 protein level difference between parental cell line and MAGEB3 knock out clones**

Because MAGEB3 knock out clones still have the mRNA level of MAGEB3, to confirm these clones were truly MAGEB3 knock out clones, MAGEB3 antibody was ordered, and Western blots were performed.
The Western blot probing for RIPK1, RIPK3 and MLKL showed that MAGEB3 knock out clones do not have the endogenous level of RIPK3 (Figure 4.9), but have an equivalent level of RIPK1 and MLKL compared to the parental cell line (Figure 4.9), which was consistent with the previous results.

The Western blot probing for MAGEB3 showed multiple bands and the range was from 20 kD to 130 kD (Figure 4.9), instead of a specific band at approximately 40 kD which was given in the profile of the antibody. And there was no difference between MAGEB3 knock out clones and the parental cell line. The multiple bands may be caused by the proteins in the MAGE family are quite similar and this antibody cannot pick up MAGEB3 from multiple MAGE family proteins.

Still, the Western blot cannot confirm these clones are MAGEB3 knock out clones.

![Western blot image](image-url)

**Figure 4.9 Endogenous expression of MAGEB3 and necroptosis related proteins in AML cell lines.**

Total cell lysates of U266, MV4:11 parental cells and knock out clones from CRISPR knock out screen were analysed for expression of MAGEB3 and proteins related to TNF
mediated necroptosis. Probe of lysates with the indicated antibodies, MAGEB3, RIPK1, RIPK3, MLKL and actin. Data representative of 2 independent experiments.

4.7.6. cDNA sequence confirmed the MAGEB3 knockout

To confirm these clones are MAGEB3 knock out clones, cDNA sequence was performed on parental cells, clone 9, 11, 12 from the CRISPR screen. The results showed the alignment of the sequence result of all the samples. The MAGEB3 lane stands for the parental cell line, which is similar to the cDNA profile in NCBI. The sequence results of clone 9, 11 and 12 showed that they all miss several base pairs (GTTCGTGATGAAGGCTT) (Figure 4.10), which was the gRNA from CRISPR library. The missing base pairs confirmed that the CRISPR/Cas 9 system had effect on the genome of the picked-up cells and it would interfere with the translation of the target gene. And the sequence results also indicated that clone 9 was different clones from clone 11 and 12, because the sequence results were different at the gRNA binding site. This result confirmed that the clones got from the CRISPR knock screen were MAGEB3 knock out clones.

![Figure 4.10 Sequence results showed that the clones were MAGEB3 knock out clones](image)

cDNA alignment of MAGEB3. Comparison of MAGEB3 cDNA from MV4;11 parental cells and MAGEB3 knock out clones. Yellow labels highlight the mismatch base pairs.
4.7.7. Knock out MAGEB3 in another human AML cell line, U937, cannot lead to necroptotic resistance

To confirm whether the effect that knock out MAGEB3 will downregulate the endogenous RIPK3 level and lead to necroptotic resistance is cell line specific or not, another human AML necroptotic sensitive cell line, U937 was chosen.

To confirm the U937 kept by our group was sensitive to necroptosis, cell death assays were performed on U937 cells and sorted into living and dead cell populations using flow cytometry. From the cell death results, 1 µM of birinapant and 10 µM of IDN can kill these cells by approximately 65%, compared to the control treatment, and the cell death rate was 15%. This result indicated that the U937 cells we had was sensitive to necroptosis, and can be used to test the effect of knock out MAGEB3.

From the previous experiments, plasmid for Cas9 and gRNA of MAGEB3 were ready to use, so the U937 was infected with Cas9 and either GeCKO gRNA of MAGEB3 or self-designed gRNA of MAGEB3, then selected for the biomarkers on the plasmid. After selection, 4 clones of each gRNA were chosen for further test.

To check whether knock out of MAGEB3 will lead to necroptotic resistance in U937 cells, cell death assays were performed and sorted into living and dead cell populations using flow cytometry. The control cells were U937 cells reconstituted with Cas9 and empty gRNA plasmid, and the cell death results showed that 1 µM of birinapant and 10 µM of IDN could kill these cells by approximately 60% (Figure 4.11A), which was close to the parental cell line. This result indicated that Cas9 and gRNA plasmid would not change the features of these cells.

The cell death result of GeCKO MAGEB3 knock out clones showed that all of these clones responded to necroptotic stimuli, and the cell death rates of treatment with 1 µM of birinapant and 10 µM of IDN were all near 60% (Figure 4.11B), which was close to the control cells. The cell death result of self-designed MAGEB3 knock out clones behaved the same way; the cell death rates were also approximately 60% (Figure 4.11A). All these results showed that knock out MAGEB3 in U937 cells would not lead to the resistance of necroptosis.
These results may be caused by several reasons: U937 cells have no endogenous level of MAGEB3, which was not widely expressed in human AML; knock out MAGEB3 in U937 cells is not related with the downregulation of endogenous RIPK3; or knock out MAGEB3 will downregulate RIPK3 in U937 cells but not enough to reach the threshold of necroptotic resistance.

To validate the hypothesis above, Western blots were performed to check the protein level of MAGEB3, RIPK1, RIPK3 and MLKL.

The Western blot probing for MAGEB3 showed that in U937 parental cell line, it has an endogenous protein level of MAGEB3 (Figure 4.11C). However, as mentioned before, the antibody for MAGEB3 was not good, and it will give several unspecific bands, which made it hard to tell which band stands for MAGEB3. And these were no difference between GeCKO MAGEB3 knock out clones and parental cells. In this Western blot, it was hard to tell whether the knockout of MAGEB3 in U937 cells was successful.

The Western blot probing for RIPK3 showed that all MAGEB3 knock out clones had an almost equivalent level of RIPK3 compared to the parental cells (Figure 4.11C), which was contrary to the MV4;11 cells. In MV4;11 cells, knock out MAGEB3 will downregulate the endogenous RIPK3 level, which may be the reason of necroptotic resistance.

The Western blot probing for RIPK1 and MLKL showed that all MAGEB3 knock out clones had the same level of RIPK1 and MLKL as the parental cells (Figure 4.11C), which indicated that knock out of MAGEB3 would interfere with other critical proteins in necroptotic pathway.

These results showed that knock out MAGEB3 would not downregulate the endogenous level of RIPK3 and lead to necroptotic resistance in U937 cells.
Figure 4.11 Cell death assay and Western blots showed that knock out MAGEB3 in U937 was not related to necroptotic resistance

(A) Cell death assays in U937 control cells and MAGEB3 knock out clones were performed using different drugs combinations. Cells were treated with 1 µM birinapant in the presence or not of 10 µM of IDN, and 20 ng/mL TNF. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM. (B) Total cell lysates of MV4:11 cells U937 parental cells and MAGEB3 knock out clones were analysed for expression of MAGEB3 and proteins related to TNF mediated necroptosis. Probe of lysates with the indicated antibodies, MAGEB3, RIPK1, RIPK3, MLKL and actin. Data representative of 2 independent experiments.

4.8 Validation of VWA7

4.8.1. Von Willebrand factor A domain containing 7 (VWA7)

VWA7 is an isoform of von Willebrand factor, which is a blood glycoprotein involved in hemostasis. The dysregulation or loss of function of von Willebrand factor may lead to
Many diseases, such as von Willebrand disease, thrombotic thrombocytopenic purpura, Heyde's syndrome, and possibly haemolytic-uremic syndrome[100].

4.8.2. The result of knock out VWA7 in MV4;11 cells did not show necroptotic resistance

To validate the result that knock out VWA7 in MV4;11 will lead to necroptotic resistance in MV4;11 cells, gRNA from GeCKO library and self-designed gRNA were used to knock out VWA7 and cell death assays were performed on selected clones.

The result of GeCKO gRNA clones showed that all four clones were sensitive to necroptotic stimuli, 1 μM birinapant and 10 μM IDN killed all the clones by approximately 65 % (Figure 4.12A), which was similar to parental cells. The result of self-designed gRNA clones also gave the same result (Figure 4.12Bd); all of the four clones were sensitive to necroptotic stimuli. All these results showed that knock out VWA7 in MV4;11 cells would not cause necroptotic resistance. The previous result may be caused by an off-target effect.
Figure 4.12 Cell death assay showed that knock out VWA7 in MV4;11 was not related to necroptotic resistance

(A) Cell death assays in MV4;11 control cells and VWA7 knock out clones were performed using different drugs combinations. Cells were treated with 1 µM birinapant in the presence or not of 10 µM of IDN. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=2 independent experiments. Values represent means ± SD.
5. Discussion

5.1. Overexpression of necroptotic related proteins in KG-1 human AML cells

KG-1, a human AML cell line derived from an erythroleukemia that evolved into acute myelogenous leukemia has been used in the group as a leukaemia cell line which expresses high levels of MDR1 and thus is resistant to apoptosis induced by several anti-cancer agents. Work in our laboratory show that independent of MDR1 expression, KG1 cell line is also resistant to necroptosis. Understanding the mechanism of KG-1’s necroptotic resistance will provide further insights of how necroptosis is regulated in AML and how this new type of cell death may be best explored for the treatment of cancer patients. To investigate the mechanism of resistance to necroptosis, we initially took an overexpression approach with necroptotic related proteins (RIPK3 and MLKL) in KG1 cells. This strategy would allow us to determine whether reconstitution of these proteins is able to sensitisce cells to necroptosis stimuli. Our data show that overexpression of RIPK3, was able to overcome necroptosis resistance in KG1 cells. For MLKL, the result of Western blot showed that wild-type MLKL was not successfully overexpressed in the KG-1 cells. Interestingly, KG1 cells do express RIPK3 protein, thus the resistance to necroptosis cannot be associated with lack of RIPK3. Therefore, we hypothesise that necroptosis resistance in KG1 may be associated with:

(1) Existence of an intermediate inhibitor of RIPK3. The overexpression system would induce high levels of RIPK3 that could overcome the effect of those inhibitors allowing RIPK3 to recruit and activate MLKL to induce necroptosis.

(2) KG1 cells have RIPK3 mutations that lead to production of a non-functional protein. Overexpression of RIPK3 replaces the endogenous defected RIPK3 allowing necroptosis signalling to continue.

(3) The KG-1 RIPK3 protein level is not high enough to continue the necroptotic signal. Overexpression of RIPK3 make the RIPK3 protein level high enough to continue necroptotic signal.

Research data showed that several types of cancers have a high percentage of RIPK3 mutation; the mutation percentage of Uterine Cancer is 3.21%; the mutation percentage of Melanoma is 3.35%; and the mutation percentage of AML is approximately 0.5% (TCGA Pancan 2018). Thus, it is plausible to imagine that mutations in RIPK3 are present.
in KG1 cells. To confirm this hypothesis and determine which mutations in RIPK3 are associated with necroptosis resistance, we sequenced endogenous RIPK3 from KG1 cells.

5.1.1. cDNA sequence detects several mutant base pairs which may lead to the loss of function of RIPK3 in KG-1 cells

Studies have shown that necroptosis has an important role in the development of several cancers[101]; necroptosis can provide a micro-environment which will contribute to tumorigenesis[102]; necroptosis can release a strong immune response that may interfere with tumor progression[103]. Necroptosis plays a different role which depends on the types and stages of cancer[20, 80], and recently, studies showed that different types of cancers trend to develop necroptotic resistance to survive[20]. RIPK3, as a critical necroptotic relate protein, is frequently downregulated or mutated in different cancers[104]. The downregulation of RIPK3 may be caused by different oncogenes or DNA methylation[104, 105]. Studies have shown that the absence of RIPK3 contribute to cancer development [105, 106]. RIPK3 has two domains; at the N-terminal is the kinase domain, which contributes to the auto-phosphorylation and phosphorylation of MLKL[107]; at the C-terminal is the RHIM domain[108], which contributes to the interaction which other RHIM domain containing proteins, such as RIPK1, or other proteins which do not lead to the activation of the kinase domain[109]. And, this loss of function of either domain will stop the necroptotic signalling[110].

In this study we show that necroptosis resistant KG1 cells have a mutation on the start codon of RIPK3(Figure 3.5A and C) in the region which is important to normal function of RIPK3. Because this region is important for RIPK3 normal translation of coding for the kinase domain of RIPK3. This mutation may lead to a loss of function form of RIPK3. There is also a mismatch at base 1201, which will not change the result of the translation of the RIPK3 of KG-1 cells. However, this mismatch may somehow interfere with the structure of the mRNA and disrupt the translation of RIPK3. This mismatch also needs further research to confirm whether it will interfere with the normal function of the KG-1 endogenous RIPK3. Since this specific mutation has not been previously reported, it would be interesting to investigate whether this mutation can also be found in other AML cell lines that are also resistant to necroptosis such as HEL cell line. The validation of this mutation as a predictor of necroptosis resistance may have important implications in the
clinical use of necroptosis stimuli for the treatment of AML. Together our results suggest that RIPK3 is a critical target for AML treatment and genetic analysis.

5.1.2. The limitations of this study and further improvement

As for overexpression of MLKL in KG-1 cell line, protein analysis demonstrated that RIPK3 can be successfully overexpressed in KG1 cells but not MLKL (Figure 3.3B). Previous studies in the group have shown that this system can be used to overexpress MLKL in MLKL knock out cells, indicating the feasibility of the system used. Several different ways have been applied to select KG-1 clones, which can overexpress wild type MLKL, such as single cell sorting and clonogenic assay. Even though many approaches have been applied, it was not able to select clones which could overexpress wild type MLKL.

This situation may be caused by several reasons;
(1) In the experiments before, this plasmid was used in MLKL knock out clones to express wild type MLKL in other human cell lines. When detected on Western bots, it was easy to tell the difference between the knockout band and the overexpress band. However, this plasmid has never been used in KG-1 cells. Even though the RIPK3 plasmid worked well in KG-1 cells, it does not mean that the MLKL plasmid will work in KG-1 cells.
(2) This plasmid is leaky (could express wild type MLKL without doxycycline treatment), and overexpression of wild-type MLKL is lethal to KG-1 cells. Overexpression wild type MLKL may lead to self-oligomerisation and translocation to the cell membrane and cause cell death[111]. In this case, after puromycin selection, all the cells, which could overexpress wild type MLKL were all dead and the cells left were not able to overexpress MLKL.

As for RIPK3 cDNA sequence, there were still things that need to be improved.

Firstly, the raw sequence result gave a high pick for the mutant start codon, instead of a mixed pick, which indicated that the KG-1 cells have homozygotes of this mutant start codon, not heterozygotes. The possibility to have the same mutation on both alleles was quite low but still possible. Furthermore, it was rare to see several consecutive base pair were mutated due to the most common mutation is single base pair mutation. This
sequence result was more likely that this gene was edited by CRISPR knock-in system instead of from real patient samples. At last, the primer used to amplify the cDNA binds too closely to the start codon, which may cause several mismatches when amplified and lead to this mutation.

To overcome all these defects, we decided to do a further genome sequence on the KG-1 cells to confirm this sequence result. Here we have Michael Hildebrand from Austin hospital to help us finish the genome sequence. All the primers for genome sequence were designed. When we get the genome sequence result, we can combine the result and clone the mutant RIPK3 into RIPK3 knock out cells to determine the expression and function of the mutant protein. If the genome sequence result doesn’t not match the previous cDNA sequence, we may design new primers to correct the cDNA sequence result. Furthermore, we can relate this mutant form of RIPK3 to real patient samples to translate why some AML patient samples cannot be cured by birinapant and IDN treatment.

5.2. Identifying new regulators of necroptosis in AML.

Our previous data has shown that human AML cell line MV4;11 is sensitive to necroptosis. To try and identify new necroptosis regulators in AML we generated MV4;11 necroptosis resistant clones using a CRISPR/Cas9 library of over 19050 genes. From 20 clones obtained we observed that gRNA for MAGEB3 was present in 5 Clones. Interestingly CRISPR of MAGEB3 was associated with downregulation or even loss of RIPK3 (Figure 4.6 A and B), suggesting that MAGEB3 is an intermediate regulator of RIPK3 and its deficiency leads to necroptosis resistance.

5.2.1. The mechanism of MAGEB3 and necroptotic resistance

There is little research focusing on MAGEB3, most of which regard MAGEB3 as a tumour antigen and immune-target[112-115]. A few studies showed that MAGEB3 corelates with X chromosome inactivation and showed a nucleoli enrichment when MAGEB3 was over-expressed[99]. All these results didn’t explain the relation between the downregulation of MAGEB3 and necroptotic resistance. Recent studies showed that the MAGE family proteins can regulate proteins through either related E3 ligase or via transcriptional level. Thus, we made two hypotheses;
(1) MAGEB3 contributes to the transcription of RIPK3 and knock out MAGEB3 downregulates the mRNA level of RIPK3
(2) MAGEB3 binds a specific E3 ligase and knock out MAGEB3 lead the E3 ligase to target RIPK3

To check whether MAGEB3 regulate RIPK3 by an mRNA level, we use PCR to quantify the mRNA level of RIPK3 in MAGEB3 knock out clones. The result showed that even though there were differences between parental cells and knock out clones, all the clones have a comparable mRNA level of RIPK3 (Figure 4.8). The other way is using real-time PCR to do an mRNA quantification in MAGEB3 knock out clones. Due to the limitation of time and no example of MAGEB3 real-time PCR for us as a source of reference, we didn't do a real-time PCR. Our results showed that MAGEB3 might not regulate by transcription but it was still unclear whether knock out MAGEB3 will interfere with the translation of RIPK3.

Another possible mechanism is that MAGEB3 may regulate the endogenous level of RIPK3 via post-translation modification. It was shown before that the MAGE family protein could bind to a specific E3 ligase via their MHD domain. The hypothesis is that MAGEB3 binds to a specific E3 ligase in MV4;11 cells, when MAGEB3 was deleted, this E3 ligase binds and ubiquitinated RIPK3 and leads to the degradation of RIPK3, thus causes the necroptotic resistance. However, the proteins in the MAGE family are so similar that we may also knock out other MAGE proteins in the meantime that may play the most critical role in this regulation. And it is difficult to identify the specific E3 ligase binding to MAGEB3 with the approaches applied in the studies. Mass spectrometry might be able to identify the specific binding protein, but due to the deficiency of the MAGEB3 antibody, it was hard to purify MAGEB3 to a mass spectrometry quality.

5.2.2. MAGEB3 antibody is defective
To confirm the MAGEB3 knock out in the knock out clones, MAGEB3 antibody was ordered. However, as demonstrated before, it was hard to tell the difference between the parental cells and the MAGEB3 knock out cells due to the multiple bands and the same pattern (Figure 4.9). These Western blots result could be caused by the proteins in the MAGE family are so similar that the antibody picked all the MAGE family proteins.
Several proteins in the MAGE family are around 40 kDs, so even though MAGEB3 was knocked out, it would not show a missing band on the Western blot. The MAGEB3 knock out clones’ cDNA sequence showed that MAGEB3 was knocked out. The result showed several missing base pairs which may interfere the translation of MAGEB3 in the MAGEB3 knock out clones.

5.2.3. Knock out MAGEB3 in U937 cells does not lead to necroptotic resistance

To check if the effect of knock out MAGEB3 was cell line specific, MAGEB3 was knocked out in another human AML cell line, U937. The cell death assay showed neither the GeCKO gRNA nor the self-designed gRNA knock out clones were resistant to necroptosis (Figure 4.11A&B). Several reasons can cause it;

(1) U937 does not have an endogenous level of MAGEB3. Even though from the Western blot, it demonstrated the same pattern as the MV4;11 cells. However, it was still unclear which bands stand for MAGEB3. If U937 does not have an endogenous level of MAGEB3, knock out of MAGEB3 will lead to no effect.

(2) The MAGE3 gRNAs were not sufficient enough to knock out the MAGEB3 in U937 due to it is a different cell line from MV4;11, cDNA sequence of MAGE3 in U937 MAGEB3 knock out clones should be performed to check the knock out efficiency.

(3) MAGEB3 played a different role in U937 cells. If MAGEB3 binds to another E3 ligase and the deletion of MAGEB3 does not lead this E3 ligase to bind to RIPK3, the deletion of MAGEB3 in U937 may have other affections, instead of necroptotic resistance.

(4) It was not MAGEB3 but other MAGE family proteins which played the most crucial role in MV4;11 cells. It was hard to prove which MAGE protein cause the downregulation of RIPK3 in MV4;11 cells, due to that the MAGE protein family is a large family and the proteins in the MAGE family are not wildly expressed in different kinds of cells.

If we want to prove that knock out MAGEB3 will downregulate RIPK3 and cause necroptotic resistance, we need to knock out MAGEB3 in other types of cells which have an endogenous level of MAGEB3 and are necroptosis sensitive.
This effect was also hard to correlate with real patient samples because MAGEB3 was not wildly expressed in AML. Data from cBioPortal showed that 1 out of 77 patients has expression of MAGEB3.

5.3. Other targets may lead to necroptotic resistance
In the CRISPR knock out screen, besides MAGEB3, there are still some other targets that may lead to necroptotic resistance.

5.3.1 VWA7 may not be a regulator of necroptotic pathway
From the CRISPR knock out screen, we got one clone which contains the gRNA of VWA7. This clone still had an endogenous level of RIPK3 and MLKL, which indicated that VWA7 might be a regulator of the necroptotic pathway. However, this result cannot be repeated with either GeCKO gRNA or self-designed gRNA (Figure 4.12A and B). All the clones were dead upon necroptotic stimuli. Several reasons may cause this result;
(1) The gRNA plasmid generated in the group was not as efficient as the gRNA in the CRISPR library, and the VWA7 was not completely knocked out with our gRNAs. So, the results were not consistent.
(2) The resistance caused by VWA7 gRNA knockout was an off-target effect. The gRNA targeted a part of the necroptotic pathway. However, it was hard to tell which part the gRNA targeted, because the VWA7 clone still got an endogenous level of RIPK3 and MLKL.
(3) The clone itself was resistant to necroptosis. Because we used a cell line for the CRISPR knock out screen, in a cell line, there may be differences among cells, and there may be cells changed their features and became resistant to necroptosis after cultured in the laboratory for some time.

5.3.2. Some other targets which may lead to necroptotic resistance
In the CRISPR knock out screen, besides MAGEB3 and VWA7, there were still some targets that may lead to the necroptotic resistance and need to be proved.

As mentioned before, clone 5, 6 and 7 all contained the gRNA of TCHH, had an endogenous level of RIPK3 and MLKL and were resistant to the birinapant and IDN treatment with the absence of extracellular TNF. When cells are treated with birinapant,
the non-canonical NF-kB pathway will be activated and produce TNF. So, knock out TCHH in MV4;11 cells may cause a deficiency in non-canonical NF-kB pathway.

Clone 17 and 20 both had the gRNA of C10orf12 (chromosome 10 open reading frame 12), and they both had an endogenous level of MLKL but downregulation of RIPK3. These two clones were all resistant to birinapant and IDN treatment even with extracellular TNF. These results indicated that C10orf12 might be a regulator of RIPK3 in MV4;11 cells, thus knock out C10orf12 will downregulate endogenous level of RIPK3 and lead to necroptotic resistance.

All these hypotheses mentioned above need further prove to avoid off-target effect of the CRISPR knock out system and it needs to be determined if this effect is cell line specific or a universal effect like what has been done on MAGEB3.
6 Conclusion

AML remains a disease with a poor prognosis for many patients without many alternatives to intensive chemotherapy for treatment. The combination of SMAC-mimetic, birinapant and caspase inhibitor, IDN to induce necroptosis may be a novel way to cure AML, but there are still patient samples resistant to birinapant and IDN treatment. This thesis aimed to discover mutations of necroptotic proteins and new regulators of necroptotic pathway, trying to understand why some patient samples are resistant to necroptosis. Utilising a variety of techniques, this has been achieved by; overexpression wild type RIPK3 in KG-1 cells sensitises KG-1 cells to necroptosis; identify several mutant base pairs on KG-1 endogenous RIPK3 mRNA, especially the mutant start codon, which possibly lead to a truncated form and the loss of function of RIPK3; using CRISPR/cas9 knock out system combined with clonogenic assay to identify several proteins which may by new regulators of necroptotic pathway; and finally, approving that knock out MAGEB3 in MV4;11 cells will lead to the downregulation of the endogenous level of RIPK3 and lead to necroptotic resistance, but this effect does not exist in U937 cell line. These results may explain why some patient samples are resistant to birinapant and IDN treatment. The patient samples may have a functionless form of RIPK3, which may stop the necroptotic signal, or these patient samples downregulate the level of MAGEB3, which will lead to downregulation of RIPK3 and necroptotic resistance. All these results may also contribute to the better clinical use of the combination of birinapant and IDN treatment.
7. References


Appendix

Supplemental figures

Figure S1. Sequence result of endogenous RIPK3 from MV4;11 cells. cDNA alignment of RIPK3. Comparison of RIPK3 from MV4;11 (upper sequence) and RIPK3 coding region (lower sequence, obtained from NCBI; NCBI Reference Sequence: NM_006871.4). Red labels highlight the mismatch base pairs.
Figure S2. MV4;11 clones may die by necroptosis with extracellular TNF

(A and B) Cell death assays in MV4;11 CRISPR knock out screen clones were performed extracellular TNF (100 ng/mL). Cells were treated with birinapant (1 µM) in the presence or not of 10 µM of IDN. Cell death rate was determined by propidium iodide staining (PI) and a flow cytometry analysis 48-hour after treatment. n=3 independent experiments. Values represent means ± SEM. TS = TNF + birinapant, TSI = TNF + birinapant + IDN.
## Materials and Recipes

### Drugs, Reagents, Media and buffers

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Sourced From</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birinapant</td>
<td>Tetralogic Pharmaceuticals</td>
<td>N/A</td>
</tr>
<tr>
<td>IND-6556</td>
<td>Tetralogic Pharmaceuticals</td>
<td>N/A</td>
</tr>
<tr>
<td>Tariquidar</td>
<td>SelleckChem</td>
<td>S8028</td>
</tr>
<tr>
<td>Zosuquidar</td>
<td>SelleckChem</td>
<td>S1481</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Sigma-Aldrich</td>
<td>D9891</td>
</tr>
</tbody>
</table>

### Antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Sourced From</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLKL</td>
<td>WEHI</td>
<td>N/A</td>
</tr>
<tr>
<td>RIPK3</td>
<td>Cell Signalling Technology</td>
<td>13526S</td>
</tr>
<tr>
<td>MAGEB3</td>
<td>Invitrogen</td>
<td>PA5-70209</td>
</tr>
<tr>
<td>Phosphor-MLKL</td>
<td>Invitrogen</td>
<td>MA5-32752</td>
</tr>
<tr>
<td>RIPK1</td>
<td>Invitrogen</td>
<td>MA5-26426</td>
</tr>
<tr>
<td>Actin</td>
<td>Invitrogen</td>
<td>MA5-11869</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG</td>
<td>Invitrogen</td>
<td>A32723</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG</td>
<td>Invitrogen</td>
<td>A32731</td>
</tr>
<tr>
<td>Goat anti-Rat IgG</td>
<td>Invitrogen</td>
<td>A-11007</td>
</tr>
</tbody>
</table>

### Reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Sourced From</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco's Phosphate Buffered Saline (DPBS)</td>
<td>ThermoFisher</td>
<td>14190-144</td>
</tr>
<tr>
<td>Immobilon Western Chemiluminescent Horse Radish Peroxidase Substrate</td>
<td>Merck Millipore</td>
<td>WEKLS0500</td>
</tr>
<tr>
<td><strong>Propidium Iodide (PI)</strong></td>
<td>Sigma Aldrich 5535-16-4</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Trypan Blue</strong></td>
<td>Sigma Aldrich T8154</td>
<td></td>
</tr>
<tr>
<td><strong>1 Kb Plus DNA Ladder</strong></td>
<td>ThermoFisher 10787081</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Necrosis Factor</strong> (TNF)</td>
<td>WEHI N/A</td>
<td></td>
</tr>
<tr>
<td><strong>Trypsin-EDTA (0.5%)</strong></td>
<td>ThermoFisher 15400-054</td>
<td></td>
</tr>
<tr>
<td><strong>FCS</strong></td>
<td>WEHI</td>
<td></td>
</tr>
<tr>
<td><strong>Effectene Transfection Reagent</strong></td>
<td>QIAGEN 301427</td>
<td></td>
</tr>
<tr>
<td><strong>RNeasy Kit</strong></td>
<td>QIAGEN 74134</td>
<td></td>
</tr>
<tr>
<td><strong>Transcriptor First Strand cDNA Synthesis Kit</strong></td>
<td>Roche 04897030001</td>
<td></td>
</tr>
<tr>
<td><strong>10× TrisAcetate-EDTA Buffer</strong></td>
<td>Sigma Aldrich T8280</td>
<td></td>
</tr>
<tr>
<td><strong>Ethidium bromide (EB) solution</strong></td>
<td>Sigma Aldrich E1510</td>
<td></td>
</tr>
<tr>
<td><strong>Wizard SV Gel and PCR Clean-Up System</strong></td>
<td>Promega A9282</td>
<td></td>
</tr>
<tr>
<td><strong>Phusion High-Fidelity DNA Polymerase (2 U/µL)</strong></td>
<td>ThermoFisher F530L</td>
<td></td>
</tr>
<tr>
<td><strong>HiSpeed Plasmid Kits</strong></td>
<td>QIAGEN 12663</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Media</strong></th>
<th><strong>Sourced From</strong></th>
<th><strong>Catalogue Number</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Iscove’s Modified Dulbecco’s Medium (IMDM)</td>
<td>ThermoFisher</td>
<td>12440-053</td>
</tr>
<tr>
<td>Dulbecco Modified Eagle Medium (DMEM)</td>
<td>ThermoFisher Scientific</td>
<td>11885-084</td>
</tr>
<tr>
<td>Roswell Park Memorial Institute (RPMI) 1640 Medium</td>
<td>ThermoFisher Scientific</td>
<td>11875-093</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Buffer</strong></th>
<th><strong>Recipe</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Running Buffer</td>
<td>95% dH₂O, 5% NuPAGE MES SDS Running Buffer (NP000202, ThermoFisher Scientific)</td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>For 20L: 14L dH₂O, 4L Methanol, 2L 10× Transfer Buffer (2L dH₂O, 288g Glycine, 60.6g Tris)</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>90% dH₂O, 10% PBST, 1;20 Skim Milk Powder</td>
</tr>
<tr>
<td>SDS Lysis Buffer</td>
<td>50mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------</td>
</tr>
<tr>
<td>Western Blot Wash Buffer</td>
<td>1× PBS with 0.05% TWEEN 20 (P1379, Sigma)</td>
</tr>
<tr>
<td>1× Tris Acetate-EDTA Buffer</td>
<td>For 15L: 1.5L Tris Acetate-EDTA Buffer, 13.5L distilled H₂O</td>
</tr>
<tr>
<td>Dulbecco's Phosphate Buffered Saline (DPBS)</td>
<td>For 1L: 8.00g NaCl₂, 0.20g KCl, 0.20g KH₂PO₄, 0.10g MgCl₂·6H₂O, 2.16g Na₂HPO₄·7H₂O, 0.10g anhydrous CaCl₂, H₂O to 1 liter</td>
</tr>
<tr>
<td>Luria-Bertani (LB) Mix (liquid)</td>
<td>For 1L: 5g Yeast Extract, 5g NaCl, 10g Tryptone, ddH₂O to 1 liter. Add NaOH to make the pH of the mix to 7.0</td>
</tr>
<tr>
<td>LB Agar solid medium</td>
<td>For 1L: 5g Yeast Extract, 5g NaCl, 10g Tryptone, 15g Agar, ddH₂O to 1 liter. Add NaOH to make the pH of the mix to 7.0</td>
</tr>
</tbody>
</table>

### DNA Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RIPK3 amplify and sequence primers</strong></td>
<td></td>
</tr>
<tr>
<td>Forward 1</td>
<td>CTCCTACGGCTCGCAATTTCC</td>
</tr>
<tr>
<td>Forward 2</td>
<td>GCCTGCTGAAAGAAGTGGTG</td>
</tr>
<tr>
<td>Forward 3</td>
<td>CGAAGCAGTGTGCAACAGG</td>
</tr>
<tr>
<td>Forward 4</td>
<td>AGCAGGCACATCTTCAGATTC</td>
</tr>
<tr>
<td>Forward 4</td>
<td>CCCTACCTCAACTTGGAACAC</td>
</tr>
<tr>
<td>Reverse 1</td>
<td>TCTTGGTCCCAGTTACCCTTC</td>
</tr>
<tr>
<td>Reverse 2</td>
<td>TGGCACTCCTCTTAACTCG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>gRNA sequence</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGEB3 GeCKO gRNA</td>
<td>AAGCCTCTCCATACCAGAAC</td>
</tr>
<tr>
<td>MAGEB3 self-designed gRNA</td>
<td>TATATTGCAGCAGAAATTGT</td>
</tr>
<tr>
<td>VWA7 GeCKO gRNA</td>
<td>GCAGCTCAACTCTTCAATC</td>
</tr>
<tr>
<td>VWA7 self-designed gRNA</td>
<td>TAGTAAVTGAAGACCCATCG</td>
</tr>
</tbody>
</table>

**MAGEB3 amplify and sequence primers** |
| Forward 1 | AAGAGTACGGCTCCTGCAGCG |
| Forward 2 | GAGCAGGAAGCTCATCAG |
Reverse 1  
CTTGGCCTTGGAACACTTCTG