Understanding the role of *FAS* mutations in DLBCL

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ABSTRACT

Diffuse Large B cell lymphoma (DLBCL) is the most common Non-Hodgkin lymphoma of B and T cell lineages. It is curable by R-CHOP (Rituximab- Cyclophosphamide, Doxorubicin, Vincristine and Prednisone) in 60% of patients in which it is the first line of regimen. Therapeutic resistance is the major cause of death in patients with relapse (30-40% of the patients). Salvage chemotherapy followed by autologous stem cell transplantation is the treatment regimen for relapsed-refractory DLBCL (rrDLBCL), however this treatment is not tolerated in older or frail patients and ultimately >90% of patients with rrDLBCL die from their disease. Recent whole exome sequencing (WES) of 38 DLBCL samples taken at the time of relapse (rDLBCL) revealed that 18% of the cases had mutations in the *FAS* gene. FAS receptor is a type I transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. FAS receptor initiates extrinsic apoptosis upon binding of FAS ligand (FASL) by surrounding cells. FAS-FASL interaction plays an important role in maintaining immune homeostasis.

We hypothesized that *FAS* mutations may favour lymphoma growth *in vivo* by impairing FAS-mediated apoptosis by surrounding immune cells. We transduced an *Eμ-Myc*/Arf-null chemo-sensitive lymphoma line with either *Fas*(Y224*) (murine equivalent of *FAS*(Y232*)), *Fas* wildtype (WT) or empty vector control. We then injected these lymphoma cells into C57BL/6 syngeneic mice and monitored lymphoma growth before and after chemotherapy. We observed that the Fas mutant cells grew faster and had a delayed response to therapy when compared to controls or Fas wildtype lymphomas. However, introduction of *Fas* into the cells triggered apoptosis and resulted in poor viability and transduction efficiency. To circumvent this problem, we bred mice that have germline mutations in Fas (*lpr* mice) with *Eμ-Myc* mice that develop spontaneous lymphomas by ~3-6 months of age, thus generating primary E*μ-Myc*/Fas$^{lpr/wt}$, E*μMyc*/Fas$^{wt/wt}$ and Fas$^{lpr/wt}$ lymphomas, all on an immune competent C57BL/6 background. The goal of this project was to study the effect of *Fas* mutations in lymphoma development, lymphoma growth rate and response to chemotherapy.

We bred *Eμ-Myc* and *lpr* mice, generating 10 E*μ-Myc*/Fas$^{lpr/wt}$ and 21 E*μ-Myc*/Fas$^{wt/wt}$ lymphomas. Mice without the *Eμ-Myc* transgene (90 Fas$^{lpr/wt}$) did not develop lymphoma. The median time to lymphoma development in the primary mice generated from breeding was not significantly

different between Fas genotypes: 164 days in Eμ-*Myc*/Fas$^{lpr/wt}$ and 161 days in Eμ*Myc*/Fas$^{wt/wt}$ (p=0.943). However, when these primary lymphomas were injected into C57BL/6 mice, lymphoma engraftment was higher in the Fas mutant group, where, 8/10 Eμ-*Myc*/Fas$^{lpr/wt}$ and 8/18 Eμ-*Myc*/Fas$^{wt/wt}$ lymphomas generated lymphomas in recipient mice. Secondary Eμ-*Myc*/Fas$^{lpr/wt}$ developed aggressive lymphomas faster (49 days) compared to the Eμ-*Myc*/Fas$^{wt/wt}$ group (95 days), p=0.045. To study the response to chemotherapy, secondary Eμ-*Myc*/Fas$^{lpr/wt}$ and Eμ-*Myc*/Fas$^{wt/wt}$ lymphomas were injected into C57BL/6 mice and treated with doxorubicin, cyclophosphamide, and vincristine. There was no significant difference in the overall survival between treated and untreated control mice in both the groups (15 days in the Fas$^{lpr/wt}$ group compared to 20 days in the Fas$^{wt/wt}$ group), p=0.181. Relapse was a common phenomenon. However, survival post treatment (time to remission/relapse) was shorter in the Eμ-*Myc*/Fas$^{lpr/wt}$ cohort compared to the Eμ-*Myc*/Fas$^{wt/wt}$ cohort for mice treated with doxorubicin and cyclophosphamide (p=0.047 for doxorubicin and p=0.058 for cyclophosphamide treatments). *Fas* mutation does favour lymphoma growth when injected in C57BL/6 mice and may contribute to therapeutic resistance. There is great heterogeneity in lymphoma growth within each Fas genotype, which may be related to other factors not yet measured in this project (e.g. selection for secondary mutations (e.g.*Tp53*), expression of Bcl2). Understanding the differences in the immune responses and the interactions in the microenvironment in these two Fas genotypes will provide a better insight into the novel therapeutic targets and their clinical significance.

ABSTRAIT

Le lymphome diffus à grandes cellules B (DLBCL) est le lymphome non hodgkinien le plus commun. Il est curable par R-CHOP (rituximab-cyclophosphamide, doxorubicine, vincristine et prednisone) chez 60% des patients, ce qui constitue la première ligne de traitement. La résistance thérapeutique est la principale cause de décès chez les patients rechuteurs (30-40% des patients). La greffe de cellules souches autologues est le traitement du DLBCL réfractaire ou en rechute (rr DLBCL), cependant ce traitement n'est pas toléré chez les patients âgés ou fragiles et finalement> 90% des patients atteints de rrDLBCL meurent de leur maladie. Le séquençage récent de l'exome complet (WES) de 38 échantillons de DLBCL prélevés au moment de la rechute (rrDLBCL) a révélé que 18% des cas avaient des mutations dans le gène *FAS*. Le récepteur FAS est une protéine transmembranaire de type I appartenant à la superfamille du facteur de nécrose tumorale (TNF). Le récepteur FAS initie l'apoptose extrinsèque lors de la liaison du ligand FAS (FASL) par les cellules environnantes. L'interaction FAS-FASL joue un rôle important dans le maintien de l'homéostasie immunitaire.

Nous avons émis l'hypothèse que les mutations *FAS* pourraient favoriser la croissance des lymphomes *in vivo* en altérant l'apoptose médiée par le FAS par les cellules immunitaires environnantes. Nous avons transduit une lignée de lymphome chimio-sensible E*μ-Myc* / Arf-null avec soit *Fas* (Y224 *) (équivalent murin de *FAS* (Y232 *)), Fas “wild type” (WT) ou le contrôle du vecteur vide. Nous avons ensuite injecté ces cellules de lymphome dans des souris syngéniques C57BL/6 et surveillé la croissance du lymphome avant et après la chimiothérapie. Nous avons observé que les cellules mutantes de Fas se développaient plus rapidement et avaient une réponse retardée à la thérapie que les contrôles ou les lymphomes de type “wild type” Fas. Cependant, l'introduction de Fas dans les cellules a déclenché l'apoptose et a entraîné une mauvaise viabilité et une efficacité de transduction. Pour contourner ce problème, nous avons développé des souris qui ont des mutations germinales chez des souris Fas (*lpr*) avec des souris *Eμ-Myc* qui développent des lymphomes spontanés vers ~ 3-6 mois, générant E*μ-Myc* / $Fas^{lpr/wt}$ primaire, E*μ-Myc* / $Fas^{wt/wt}$ et les lymphomes de $Fas^{lpr/wt}$, tous avec des souris immunocompétentes. Le but de ce projet était d'étudier l'effet des mutations *Fas* dans le développement du lymphome, le taux de croissance des lymphomes et la réponse à la chimiothérapie.

Nous avons élevé des souris *Eμ-Myc* et *lpr*, en générant 10 lymphomes E*μ-Myc* / $Fas^{lpr/wt}$ et 21 E*μMyc* / $Fas^{wt/wt}$. Des souris sans le transgène *Eμ-Myc* (90 Fas^{mut}) n'ont pas développé de lymphome. Le temps de développement du lymphome chez les souris primaires n'était pas significativement différent entre les génotypes *Fas*: 164 jours chez E*μ-Myc* / $Fas^{lpr/wt}$ et 161 jours chez E*μ-Myc* / $Fas^{wt/wt}$ (p = 0,943). Cependant, lorsque ces lymphomes primaires ont été injectés dans des souris C57BL/6, la prise de greffe de lymphome était plus élevée dans le groupe mutant Fas, où les lymphomes 8/10 E*μ-Myc* / $Fas^{lpr/wt}$ et 8/18 E*μ-Myc* / $Fas^{wt/wt}$ ont généré des lymphomes chez des souris receveuses. E*μ-Myc* / $Fas^{lpr/wt}$ secondaire a développé un lymphome agressif plus rapidement (49 jours) que le groupe E*μ-Myc* / $Fas^{wt/wt}$ (93 jours), p = 0,045. Pour étudier la réponse à la chimiothérapie, des lymphomes secondaires E*μ-Myc* / $Fas^{lpr/wt}$ et E*μ-Myc* / $Fas^{wt/wt}$ ont été injectés dans des souris C57BL/6 et traités avec de la doxorubicine, du cyclophosphamide et de la vincristine. Il n'y avait pas de différence significative dans la survie globale des souris témoins non traitées dans les deux groupes (15 jours dans le groupe $Fas^{lpr/wt}$ par rapport à 20 jours dans le groupe $Fas^{wt/wt}$), p = 0,181. La rechute était un phénomène commun. Cependant, la survie posttraitement (délai de rémission / rechute) était plus courte dans la cohorte E*μ-Myc* / $Fas^{lpr/wt}$ comparée à la cohorte E*μ-Myc* / $Fas^{wt/wt}$ pour les souris traitées à la doxorubicine et au cyclophosphamide (p = 0,047 pour la doxorubicine et p = 0,058 pour la cyclophosphamide). traitements). La mutation *FAS* favorise la croissance du lymphome lorsqu'elle est injectée chez la souris C57BL/6 et peut contribuer à la résistance thérapeutique. Il y a une grande hétérogénéité dans la croissance des lymphomes dans chaque génotype *Fas*, qui peut être liée à d'autres facteurs non-encore mesurés dans ce projet (par exemple sélection pour des mutations secondaires (par exemple *Tp*53), expression de Bcl2). Comprendre les différences dans les réponses immunitaires et les interactions dans le microenvironnement dans ces deux génotypes Fas permettra de mieux comprendre les nouvelles cibles thérapeutiques et leur signification clinique.

Preface and Contribution of Authors

The following is my Master's thesis titled "Understanding the role of *FAS* mutations in DLBCL" which is composed of 3 chapters in accordance to the guidelines outlined by the Department of Graduate and Postdoctoral Studies. The first chapter is the detailed 'Literature review', chapter 2 consists of 'Introduction, Hypothesis, Objectives, Methods, Results, Figures and Discussion' and the final chapter 3 is the 'Conclusion and Summary'. The contributions of each author are discussed below.

Most of the experiments and data analysis were performed by Maanasa Venkataraman.

Previous experiments discussed in the introduction and used as basis for this project were performed by Stephanie Totten and Dr. Maryse Lemaire.

Mice injections were done by Yvhans Chery or Kathy-Ann Forner. The animal quarters staff Darlene helped with mice housing and monitoring. Veronique Michaud helped initially with the Ultrasound VEVO machine.

All the mice experiments were assisted by Jibin Zeng. Dr. Claudia Weaver helped with the figures. Dr. Nathalie Johnson helped with the analysis of the data.

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Abbreviations

ABC: Activated B cell like
APAF-1: Apoptotic protease activating factor 1
AID: Activation-induced deaminase
ALPS: Autoimmune lymphoproliferative syndrome
BAD: Bcl-2-associated death promoter
BAK: Bcl-2 homologous antagonist/killer
BAX: Bcl-2-associated X protein
BCL2: B-cell lymphoma 2
BCL6: B-cell lymphoma 6
BCL-XL: B-cell lymphoma-extra large
BCR: B cell receptor
BCS: Body condition score
BH3: Bcl-2 homology domain 3
BID: BH3 interacting-domain death agonist
BIK: Bcl-2 interacting killer protein
BIM: Bcl-2 interacting mediator of cell death
BL: Burkitt's lymphoma
CAD: Caspase-activated deoxyribonuclease
CAR-T: Chimeric antigen receptor -T cells
CD: Cluster of differentiation
CRD: Cysteine-rich domain
CTL: Cytotoxic T lymphocyte
DD: Death domain
DISC: Death-inducing signaling complex
DLBCL: Diffuse large B cell lymphoma
EZH2: Enhancer of zeste homolog 2
ER: Endoplasmic reticulum
FADD: FAS-associated death domain protein
FAS: Human FAS Protein
FAS: Human FAS gene
Fas: Mouse FAS transgene
Fasl: Mouse FAS ligand transgene
Fas: Mouse FAS gene when part of genome
Fasl: Mouse FAS ligand gene when part of genome
FCM: Flow cytometry
FL: Follicular lymphoma
GC: Germinal center
GCB: Germinal centre B-cell-like
GFP: Green-fluorescent protein
GZMB: Granzyme B

ICAD: Inhibitor of caspase-activated deoxyribonuclease ICD: Immunogenic cell death iMEF: Irradiated murine embryonic fibroblast
IgH: Immunoglobulin heavy locus
IP: Intraperitoneal
IPA: Ingenuity Pathway Analysis
IV: intravenous
IVIS: In vivo imaging system
MCL-1: Induced myeloid leukemia cell differentiation protein
MEF: Murine embryonic fibroblast
MOMP: Mitochondrial outer membrane permeabilization
NHL: Non-Hodgkin lymphoma
OS: Overall survival
PARP: Poly (ADP-ribose) polymerase
PCD: Programmed cell death
PFS: Progression free survival
PUMA: p53 upregulated modulator of apoptosis
R-CHOP: Rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone rrDLBCL: Relapsed/refractory diffuse large B cell lymphoma
TLy: Transformed lymphoma
TM: Transmembrane
TNF: Tumor necrosis factor
TNF-R1: Tumor necrosis factor receptor 1
TP53: Tumor protein p53
WES: Whole exome sequencing

Chapter 1: Literature Survey

1.1 Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma (NHL) is a group of heterogenous haematological malignancies arising from B, T, and natural killer cells, which have different histological and biological characteristics. It is one of the most common cancers in North America, accounting for about 4% of all cancers[1,2]**.** According to the American Cancer Society, the risk of people developing NHL is 1 in 47. About 80-85% of all NHL arise from B cells, 10-15% from T cells while NHL arising from natural killer cells are rare[3]. Genetic tools like gene expression profiling have led to the discovery of novel pathways that are clinically relevant in the malignant transformation of lymphoma[4]. Although a definitive cause cannot be defined for a lymphoma, multiple risk factors are involved including age, gender, exposure to certain drugs, chemicals and radiations, presence of certain infections, autoimmune disorders, single or multiple mutations and genetic events[3].

Ninety percent of lymphomas are of B cell origin and develop because of errors that occur during the different stages of B cell development[3]. B cell development starts in the bone marrow with V(D)J recombination of heavy and light chains of the antibody which leads to the formation of the B cell receptor (BCR). Once the BCR is expressed, mature naive B cells leave the marrow where they can circulate in the peripheral blood until they encounter antigens. Once activated by an antigen, they travel to the germinal centre of lymph nodes for further development and activation. Two distinct genetic events occur in the germinal centre- Somatic hypermutation (SHM) and class switch recombination (CSR). Activation induced cytidine deaminase (AID) acts as the catalyst for both these major events. Somatic hypermutation carries out mutations, insertions, deletions in the variable region of the immunoglobulin gene (Ig) to produce an array of antibodies against the newly encountered antigens. Class switching recombination follows by changing the heavy chain region from IgM to IgG, IgA and IgE. Class-switched B cells who have undergone SHM exit the germinal centre to become either plasma cells or memory B cells, waiting for further encounters[5]. These step by step events are controlled to ensure proper production of antibodies and proper functioning of the immune cells[5,6]. Mistakes can occur anywhere during development that may lead to aggressive lymphomas[5]. However, given that most of the genotoxic stress occurs in the germinal centre during SHM and CSR, the most common lymphomas are germinal centre B

cell lymphomas, where diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) represent 70% of all NHL[2].

1.1.1 Clinical features and treatment of DLBCL

DLBCL constitutes 30-40% of NHL and is characterized by an aggressive clinical course[7,8]. Patients with DLBCL can exhibit "B-symptoms" like weight loss, fever, and night sweats. There is a diversity in the clinical presentation which depends on the extra nodal sites in which the lymphoma cells are present. The most common extra nodal sites during diagnosis include the gastro-intestinal tract, spleen, liver, kidney, thyroid, testis, and bone marrow[9,11].

The diagnosis of DLBCL relies upon the biopsy of a lymph node and/or tissue from an extra nodal site. Neoplastic cells typically have large nuclei, twice as big as the size of a regular lymphocyte[12]. There are many cases that are biologically and clinically heterogenous and cannot be subclassified, known as DLBCL, not otherwise specified (DLBCL, NOS)[13]. They might arise *de novo* or could also transform from an indolent lymphoma, the most common being FL. They consist of a mixed population of centroblasts, immunoblasts and centrocytes mixed with T cells and histiocytes[12,13]. Using recent gene expression profiling, DLBCL can be classified as germinal centre B cell like (GCB) DLBCL or activated B cell like (ABC) DLBCL according to the cell of origin (COO), with the ABC type being associated with worse outcomes[14,15]**.** ABC DLBCL consists of mature B cells that have gone through the germinal centre reaction and are poised to undergo plasmablastic differentiation, i.e. to differentiate into either plasma cells or memory B cells. Similarly, GCB DLBCL consists of mature B cells in the germinal centre[14,16]. Immunohistochemistry and flow cytometry help to make the diagnosis of DLBCL and subclassify it into different molecular subtypes (ABC versus GCB)[13,17].

Once the diagnosis of DLBCL is confirmed, the patient undergoes staging which includes imaging (CT scans) and a bone marrow biopsy to help establish the disease prognosis and treatment[14,18]. The Ann-arbor staging system is commonly used in clinics and takes the symptoms into account[19]. Stage 1 is the involvement of a single lymph node or a single extra nodal site. Stage 2 is defined as the involvement of 2 or more nodes on the same side of the diaphragm. Stage 3 is defined as the involvement of 2 or more nodes on both sides of the diaphragm. Stage 4 is defined

as diffuse involvement of extra nodal sites and the association of lymph nodes. Stages 1 & 2 are considered early stage DLBCL whereas stages 3 & 4 are considered advanced stage DLBCL[18,19].

The International Prognostic Index (IPI) is a major clinical tool used to predict disease outcome in patients with DLBCL[14,15,20]. Before the introduction of rituximab in the treatment regimen, five prognostic factors were used: age, lactate dehydrogenase (LDH) levels, number of extra nodal sites, staging and Eastern Cooperative Oncology Group performance status (ECOG). The negative prognostic factors at diagnosis include age > 60 years, high serum lactate dehydrogenase levels, extra nodal sites > 1, ECOG ≥ 2 and stage 3 or 4 of the disease. According to this prediction, patients were categorized into 4 outcome groups with 5-year progression free survival from 26% to 73%[14]. After the introduction of rituximab, the IPI has been modified to include the extra nodal involvement of bone marrow, CNS, lungs, liver/gastrointestinal tract as a negative factor instead of the number of extra nodal sites. The modified IPI was obtained using data from National Comprehensive Cancer Network (NCCN-IPI) and they were able to better categorize patients into two rather than four outcome groups, since rituximab had 15% positive effect in the 5-year OS[14,15].

Even in advanced stage disease, chemo-immunotherapy is the standard therapy for DLBCL. The CHOP chemotherapy regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) given every 21 days for 6 cycles has been the gold standard of treatment for patients with DLBCL for > 25 years. Almost 75% of these patients are diagnosed with stage 3&4 DLBCL[14]. The addition of the monoclonal anti-CD 20 antibody rituximab to the CHOP backbone has improved the overall survival (OS) of DLBCL patients by over 15% and thus has become the standard of care since 2001 [18,21,22,23]. R-CHOP cures ~60% of the patients with DLBCL[21,22]. However, refractory/relapsed DLBCL is observed in ~30-40% of cases [22,24]. Other intensive agents that are more toxic than R-CHOP have failed to show any survival advantage over R-CHOP, thus patients with high-risk features are encouraged to enrol in clinical trials to improve their outcome [22,25,26].

1.1.2 Biology of DLBCL

Beyond cell of origin classification, large scale sequencing studies have helped improve the biology of DLBCL. Each molecular subtype of DLBCL has different oncogenic pathways that

drive the tumor biology. The most common recurrent somatic point mutation observed in GCB is in the gene histone methyl transferase *EZH2* (~20%) which leads to a gain of function of the EZH2 protein, which in turn increases methylation of histone 3 and this is observed to promote lymphoma progression by transcriptionally silencing regulator genes[27,28,29]. This event, in association with the transcriptional repressor BCL6, mediates the majority of GCB DLBCL cases[30]. Increased cellular metabolism and growth is also observed in GCB, which may be due to constitutive activation of PI3K/MTOR pathway with loss of PTEN expression, due to deletion of the tumor suppressor *PTEN*[31]. The most negative prognostic factor for GCB is the overexpression of anti-apoptotic *BCL2* due to t(14:18). In contrast, the ABC subtype is characterized by the constitutive activation of NF-κB pathway which promotes various cellular events such as proliferation, survival, and inhibition of apoptosis[14,33]. Approximately 20% of cases harbor mutations in the B cell receptor (BCR) pathway which leads to activation of BCR signalling, which in turn enhances the NF-κB pathway. Overexpression of BCL2 is observed in ABC as well but is done through different mechanisms than GCB. Transcriptional upregulation and gene amplification increase the expression of BCL2 rather than the translocation of *BCL2*[34].

The tumor microenvironment plays an important regulatory role in DLBCL growth and proliferation. Tumor cells are surrounded by other immune cells, lymphocytes (CD4+ T cells, CD8+ T cells, B cells and NK cells), inflammatory cells, blood vessels and extracellular matrix[35]. Targeting the host-tumor interaction in these hematopoietic malignancies by various novel drugs has provided a promising future in cancer research. There is heterogeneity observed in the cellular composition of the microenvironment in each subtype of DLBCL[35,36]. The Lymphoma/leukemia molecular profiling project categorized the microenvironment of DLBCL according to gene expression signatures: stromal 1, associated with a favourable outcome, and stromal 2, associated with an unfavourable outcome[37]. Stromal 1 consists of extracellular matrix deposition and infiltration of macrophages (CD68), whereas stromal 2 consist of a high density (CD34) of blood vessels and angiogenesis[35,37]. Recent studies of DLBCL independent of COO show recurrent genetic lesions in immune recognition which aids the tumor in escaping from immune surveillance[14]**.** Approximately 29% of mutations or deletions are observed in the *B2M* gene, which leads to inactivation of the β_2-microglobulin gene and 21% of inactivating mutations are in the *CD58* gene. These genes are critical in immune recognition of tumor cells by cytotoxic T cells and

Natural killer cells (NK) respectively[38]. One recent study highlighted the use of CD4+/CD25+/FOXP3+ T regulatory cells (T_{regs}) as a negative prognostic marker in NHL. T_{regs} allow tumor cells to proliferate by suppressing the development and cytokine production of cytotoxic T cells, thereby inhibiting the anti-tumor immune response[36]. Another key finding is the upregulation of Programmed cell death ligand (PD-L1) by malignant cells in lymphoid malignancies and cytotoxic T lymphocyte antigen 4 (CTLA-4) and PD-1 by tumor infiltrating lymphocytes[39]. These immune check points inhibit the anti-tumor response of the cytotoxic T lymphocytes[40].

DLBCL has an array of genomic alterations that are associated with an inferior/poor outcome, including deregulation of potent oncogenes and tumor suppressor genes such as *MYC*, *BCL2* and *P53*[8,41]. The oncogene *MYC* is rearranged in ~10% of DLBCL and MYC protein is expressed by IHC in 30% of DLBCL cases [14,8,42]. *MYC* is located at the chromosome locus 8q24.21, which codes for a helix-loop-helix transcription factor that controls cell growth and survival, cell cycle progression, metabolism, and apoptosis. Its initial involvement in causing malignancy was described in Burkitt's lymphoma (BL), where the translocation t(8;14)(q24;q32) leads to the juxtaposition of *MYC* with the *IgH* enhancer, which in turn leads to overexpression of *MYC*[42]. MYC deregulation (translocation or protein expression) is associated with a poor outcome, especially when BCL2, a potent inhibitor of apoptosis, is also co-expressed. The outcome is very poor when there is the presence of concurrent translocations in *MYC* and *BCL2,* which is currently classified as high-grade lymphomas, so-called 'double hit' lymphomas (HGBL-DH)[43]. The overexpression of the anti-apoptotic protein BCL2 is a common event in DLBCL and t(14:18) is found in 30-40% of GCB DLBCL. This translocation event is considered a negative prognostic factor and is associated with a poor outcome in GCB[14]. *TP53* is a well-known tumor suppressor gene that has been involved in many cancers, including lymphoma. TP53 plays multiple roles including regulation of cell cycle progression, apoptosis, and DNA repair. Loss of TP53 or mutations in *TP53* are associated with poor overall survival (OS) and progression free survival (PFS) in patients treated with RCHOP[41]. *TP53* alterations are also considered one of the important genetic events underlying the transformation of an indolent lymphoma into aggressive DLBCL[41].

All three oncogenes *MYC*, *TP53* and *BCL2* have been extensively studied in lymphoma mouse models[44]. The potent role of *MYC* was first described in transgenic mice in 1985[45]. One of the best studied murine models for BL and DLBCL is the *Eμ-Myc* model, where *Myc* is placed under the control of the *IgH* enhancer, leading to Myc overexpression in B cells [44,45,46]. The *Eμ-Myc* model has faithfully recapitulated the chemo-resistant phenotype observed in human MYC-driven lymphomas that have mutations in *TP53* or over-expression of BCL2[47]. Thus, it is a good model to assess if a gene influences lymphoma growth and chemosensitivity.

1.1.3 Biology of relapsed DLBCL

While the biology of diagnostic DLBCL has been extensively studied, very little is known about the biology of this disease at the time of relapse. To understand the genetic events that are enriched at the time of relapse, we performed whole exome sequencing (WES) in 38 DLBCL samples obtained from patients after their disease relapsed following RCHOP and subsequent therapies[48,49]. The most frequent mutations that were independent of COO were *TP53*, *MLL2* and *FAS*. The genes that were commonly mutated in ABC relapsed or refractory DLBCL (rrDLBCL) included *PIM1*, *MYD88* and the most commonly mutated genes in GCB rrDLBCL included *STAT6*, *FOXO1* followed by *BCL2* and *EZH2*. Paired biopsies obtained at diagnosis and relapse were used to study the clonal expansion of some mutations after treatment. Mutations in *TP53, FOXO1, MLL3 (KMT2C), CCND3, NFKBIZ*, and *STAT6* emerged as the top genes being implicated in therapeutic resistance because there was evidence of clonal selection and a higher frequency at relapse[50,51,52]. Studying the tumor biology at relapse would aid our understanding of potential biomarkers, which could be targeted by either existing therapies or novel therapies that are in development.

1.1.4 Treatment of relapsed DLBCL

Despite this improved knowledge of tumor biology, the outcome of patients with rrDLBCL is very poor[53]. Salvage chemotherapy followed by autologous stem cell transplant can provide long term remission in 30% of patients who are still chemo-sensitive at the time of relapse[22,23,24]. However, many patients are too old or frail to tolerate the toxicity of this treatment and ultimately ~90% of patients with rrDLBCL die from their disease[24]. Clinical trials offer patients access to newer drugs that kill cancer cells using different mechanisms of action. These include drugs that target specific pathways or proteins, e.g. ibrutinib targets BCR signaling and venetoclax targets

BCL2. These and other targeted therapies such as lenolidomide, idelalisib and bortezomib have demonstrated clinical activity in a subset of patients with rrDLBCL[54,55,56]. Other approaches include using immunotherapy with immune check point inhibitors such as anti-PD1 drugs or using engineered chimeric antigen receptor (CAR) T cells that target CD19. These can also provide remissions in a subset of rrDLBCL patients[57,58]. Thus, there is a rationale to study the tumor biology at relapse to understand the pathways/mechanisms that drive tumor cells and promote immune evasion in rrDLBCL. This additional knowledge may help design more effective treatment regimens for high-risk DLBCL patients that are not cured with RCHOP[55].

1.2 Apoptosis

The term apoptosis was first coined in 1972 to describe a morphological phenomenon of cell death. Apoptosis or programmed cell death (PCD) is characterized by morphological and structural changes as well as biochemical mechanisms[59,60,61,62]. It is considered a vital phenomenon for maintaining homeostasis in tissues, development, and senescence. It is a defense mechanism as infected cells are eliminated from the body through apoptosis[59,63]. Apoptosis can be achieved through several mechanisms and pathways and by different stimuli. For instance, irradiation can cause DNA damage which in turn activates TP53 mediated apoptosis[59]. Tumor necrosis family (TNF) like FAS or other receptors are expressed on the cell surface and initiate apoptosis through corresponding ligand binding from neighbouring cells[59]. Apoptosis is characterized by structural changes such as membrane blebbing, cell size shrinking, nuclear material fragmentation and chromatin condensation. These fragments are digested by phagocytes or by their own lysosomes[60,61]. When the cell shrinks, the cytoplasm gets dense and the organelles become more tightly packed. Following which the membrane blebbing occurs, small pieces of apoptotic bodies are formed, each with tightly packed organelles enclosed with a plasma membrane. These bodies are then ingested by phagocytes and degraded by their phagolysosomes[59,63]. Apoptosis is an ATP dependent pathway controlled by various factors[60].

There are three main apoptotic pathways: intrinsic, extrinsic and T cell induced perforin/Granzyme B-dependent[60]. Until recently, there was a clear distinction between these pathways but now it has been shown that they are interconnected and influence each other.

Ultimately all three pathways lead to caspase-3 activation and all the structural changes associated with apoptosis (as mentioned previously) and cell death. Granzyme A activates a caspase independent pathway which ultimately cleaves and damages the DNA of the cell[59,62,64].

Caspases are a family of conserved cysteine rich proteins that are normally inactive in the cell but have proteolytic function once activated[59,60,62,63]. Caspases can be further classified ,based on their position in the apoptotic signalling cascade, into initiator caspases (2,8,9,10), effector caspases (3,6,7) and inflammatory caspases (1,4,5)[62,63]. The initiator caspases initiate the process of apoptosis by cleaving the downstream effector caspases. The inactive pro caspase monomers in the cell get cleaved and activated in response to any apoptotic stimuli. Caspase 3 is an important effector caspase since it is activated by almost all the initiator caspases. The effector caspases cleave various cellular substrates like poly ADP-ribose polymerase (PARP) and other target proteins required to produce the morphological changes required for cell death. Caspase 3 activates the nuclease caspase activated deoxyribonuclease (CAD) by cleaving its inhibitor leading to fragmentation of chromosomal DNA[62,65,66].

1.2.1 Intrinsic Apoptosis

Intrinsic apoptosis is also known as mitochondrial apoptosis whereby a sequence of events leads to release of cytochrome C from the mitochondria, an irreversible step that commits the cell to death. It is initiated by various stresses like radiation, hypoxia, DNA breaks, or chemotherapy agents, and is mediated by the tumour suppressor p53[59,60,62]. Intrinsic apoptosis is driven by the interplay and modulation of the BCL2 family of proteins. The apoptotic events lead to formation of mitochondrial outer membrane permeabilization (MOMP), leading to loss of transmembrane potential, thus releasing cytochrome C. This interacts with the apoptotic protease activating factor (APAF-1) and procaspase 9 forming the apoptosome. The activated caspase 9 cleaves and activates effector caspase 3, thus initiating further downstream apoptotic events[59,62,67].

The BCL2 family of proteins can be both pro-apoptotic or anti-apoptotic in nature. Some of the pro-apoptotic proteins include BAX, BAK, BID, BIM, etc and some of the anti-apoptotic proteins include BCL2, BCLW, BCLXL and MCL1. They play an important role in deciding the fate of the cell, i.e. whether it should undergo apoptosis or abort the process[59,60,62]. The major anti-

apoptotic protein BCL2 is over-expressed in most B cell lymphomas[14,67]. BCL2 family proteins can be classified into two pro-apoptotic groups: effectors (BAX and BAK) and activators (BID and BIM). BAX and BAK undergo conformational change and their activation by the activators causes MOMP, ultimately leading to cytochrome C release and commits the cell to apoptosis. The role of the anti-apoptotic BCL2 family of proteins is to bind to the activators and prevent them from activating the effectors, thus preventing the event of MOMP formation. There is another group of BCL2 proteins known as 'sensitizers' that are not capable of directly activating the effectors BAX and BAK. They compete for BH3 binding domain with anti-apoptotic proteins and prevent them from binding to BID and BIM. Some of these sensitizers include BAD, BIK, PUMA, NOXA and BMF. PUMA and NOXA play an important role in p53 mediated apoptosis and are pro-apoptotic in nature. Intrinsic apoptosis is modulated by the changes and balance of the BCL2 family of proteins[59,62,67].

1.2.2 Perforin/Granzyme B mediated apoptosis

Cytotoxic T lymphocytes (CTLs) initiate apoptosis through the FAS-FASL mediated extrinsic apoptotic pathway (see below)[59,68] and by secreting proteins like perforin and Granzyme A/B[64]. Perforin is a transmembrane pore forming protein secreted by CTLs to eliminate tumor and other virus-infected cells. After the pore formation, cytoplasmic granules are released into the target cells. These granules contain serine proteases like Granzyme A/B. Granzyme B cleaves and activates caspase 10 which in turn activates effector caspase 3, thus initiating the apoptotic pathway. Granzyme A induces a caspase-free apoptotic pathway by ultimately cleaving DNA[59,62,64].

1.2.3 Extrinsic Apoptosis

The extrinsic signalling apoptotic pathway involves receptor mediated response to stimuli[59,62]. It is stimulated by Tumor Necrosis factor receptors (TNF-R) such as FAS, TNFR1, DR3, DR4 and DR5. FAS is a type I transmembrane protein that is a cell surface receptor which binds to its corresponding ligand FASL from the neighbouring cells and initiates apoptosis[62,69]. Members of TNF-R family share a conserved cysteine rice cytoplasmic domain, a transmembrane domain and a cytoplasmic domain known as the 'death domain' (DD)[69]. Extrinsic apoptosis is best described by FAS/FASL and TNFR1/TNF-α models. The cell surface receptors cluster and bind to their

corresponding trimerized ligand. FASL binding leads to FAS receptor oligomerization and recruits the adaptor protein FAS associating death domain containing adaptor protein (FADD) and procaspase 8, thus forming death inducing signalling complex (DISC)[62,70]. DISC formation is an important phenomenon in extrinsic apoptosis that further controls the downstream apoptotic pathway. DISC formation causes activation of procaspase 8 into caspase 8, ultimately cleaving and activating effector caspases like caspase 6,7 and 3, and morphological changes of apoptosis. c-FLIP is an inhibitor protein that binds to FADD and caspase 8 thereby inactivating them and ultimately inhibiting apoptosis[62,69,70].

1.2.3-1 FAS-Death receptor

Fas/Apo-1/CD95 is a cell surface type I transmembrane protein belonging to Tumor necrosis factor receptor (TNF-R) family of proteins[62,69]. It is located at chromosome 10 in humans. It consists of 9 exons which encode for an N-terminal extracellular domain, one transmembrane domain (coded by exon 6) and a cytoplasmic domain which contains the death domain (DD) (Figure 1). There are 3 cysteine rich domains (CRD) which are glycosylated in the extracellular region and aid in FASL binding from the neighbouring cells[62,69]. The C-terminal tail consists of 6 α-helices which play an important role in DISC formation and apoptosis[70]. FAS spontaneously assembles into trimers on the cell membrane before binding to their corresponding FAS ligand. FASL is a type II transmembrane protein which belongs to the tumor necrosis family (TNF). FAS is predominantly expressed on T and B lymphocytes and natural killer cells in various tissue types[71]. FASL is constitutively expressed on activated T cells and natural killer cells and is abundantly available in the thymus, liver, heart, and kidney[72].

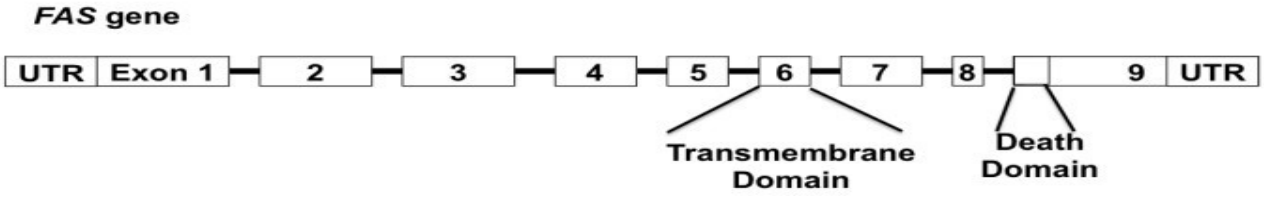

Figure 1. *FAS* gene structure[62]

1.2.3-2 FAS mediated apoptosis

FAS initiates apoptosis by binding to its ligand FASL from the neighbouring cells and causing cell death[59,70]**.** The key role of FAS is to maintain immune cell homeostasis by FASL expressed in T and NK cells binding to FAS expressed on infected or auto-reactive B or T cells[71]. The clustered FAS binds to trimerized FASL and this binding causes recruitment of adaptor protein FADD and procaspase 8 in its inactive state to the DD of FAS[70]. Together, these form the DISC complex which is the most important step of this apoptotic pathway. Formation of DISC oligomerizes and activates procaspase 8 which activates the effector caspase 3 and causes hallmark structural changes of apoptosis like nuclear fragmentation, membrane blebbing, cell shrinkage etc[59,70]. A 'crosstalk' of extrinsic and intrinsic apoptotic pathways can also occur where the activated procaspase 8 in this pathway cleaves the BCL2 family activator BID, thus initiating the mitochondrial apoptotic pathway and leading to the release of cytochrome C[59,60]**.**

1.2.4 *FAS* mutations observed in cancer and their impaired function

The most predominant apoptotic role of FAS is to eliminate cancer or virus infected cells while binding to FASL from cytotoxic T lymphocyte (CTL), apart from maintaining immune cell homeostasis[71,72]**.** Evading immune surveillance is one of the important hallmarks of cancer that has been researched recently. Many of the cancer cells develop resistance to FAS mediated apoptosis by various mechanisms[73]**.** One of the many methods is the upregulation of cFLIP inhibitory protein that blocks the formation of DISC. This leads to loss of function of FAS-FASL mediated apoptosis, thus cancer cells evade tumor immunosurveillance[74,75,76,77,78]. FAS can also be down regulated which has been studied in multiple cancer models that utilize transcriptional inactivation, promoter methylation and reduced histone deacetylation, alternate splicing and inactivating *FAS* mutations[62]**.**

Mutations in the primary structure of FAS disrupt FAS-mediated apoptosis. Missense mutations are observed only in the death domain (DD) in 5% of gastric cancers[73]. The *FAS* gene locus is deleted in ovarian cancers and their mutations might contribute to their clinical outcome[79]. MRL *lpr/lpr* mice have germline autosomal recessive mutations in *FAS* which was used to study the role of *FAS* in maintaining immune cell homeostasis[80]. Deficient FAS-FASL interactions are observed in generalized lymphoproliferative disease (*gld*) mice that have a spontaneous mutation

in FASL[80]**.** Autoimmune lymphoproliferative syndrome (ALPS) or Canale-Smith syndrome is the most common disease with heterozygous dominant *FAS* mutations[80,81]**.** The characteristic features are splenomegaly, lymphadenopathy, auto-immunity, and a higher chance of developing B cell lymphomas[11]**.** Lymphadenopathy is marked by proliferation and accumulation of T cells. Approximately 15-20% of rrDLBCL patients have *FAS* inactivating mutations[81]. In a cohort of 150 NHL patients, FAS mutations were observed in 3 MALT- type lymphomas, 9 DLBCL, 2 FL etc. Most of the mutations were directed to disrupt or alter the structure and function of the FAS receptor. 6/16 mutations were missense that led to amino acid substitution and 4/6 (D244V, E256K, L262F, and K283N) were in the death domain encoding region of FAS and were highly conserved (exon 9). For instance, D244V mutations were like those found in ALPS patients which disrupted the apoptotic signalling, since the alteration in the codon led to low frequency of binding of FAS death domain to FADD protein[82]. Whole exome sequencing of 38 rrDLBCL patients showed that *FAS* mutations are uncommon at diagnosis (7%) but more frequent during relapse[48]**.** Even though incidents of FAS mutations have been reported in lymphomas, little is known about their role in lymphoma progression and their clinical outcome. Most of the inactivating mutations are heterogenous and are found in the cytoplasmic tail DD. They have a dominant negative role since one copy of the mutated allele is enough to disrupt FAS mediated apoptosis[83]**.**

1.2.5 FAS/FASL expression and function in response to chemotherapy

Chemotherapy can induce apoptosis and non-apoptotic death like necrosis, senescence, and autophagy[84]. Chemotherapy drugs would generally initiate the intrinsic apoptotic pathway. The DNA damage response would be initiated through TP53 which activates the pro-apoptotic BCL2 family proteins[62]. However, chemotherapy can also initiate FAS-mediated apoptosis[85]. For example, doxorubicin induced FAS mediated apoptosis in primary leukemia cells *in vivo*[62,86]. In a cohort of acute myeloid leukemia (AML) patients, a positive significant correlation was found between FAS expression and response to chemotherapy[87]. Initial, preliminary experiments conducted in our lab showed that Fas expression increased in malignant B cells after doxorubicin and cyclophosphamide treatment i.e. chemotherapy engages Fas mediated apoptosis[62].

Chapter 2: Understanding the role of *FAS* mutations in DLBCL

2.1 Introduction

A key feature of cancer is the inhibition of apoptosis[76]. We recently profiled DLBCL at the time of relapse and found that 15-20% of cases had mutations in *FAS*, predicting a dysfunctional death domain[48]. These mutations are uncommon at diagnosis (7%) suggesting that they have a growth advantage or are resistant to chemotherapy and would be selected for during RCHOP. We initially confirmed prior work that mutations in *FAS*, even in the heterozygous state, are dominant-negative and impair FAS-mutated apoptosis[62]. These *in vitro* experiments were carried out in human cell lines that were transfected with *FAS*(Y232*), the most common hotspot mutation (replacing stop codon for tyrosine residue at amino acid 232) producing a truncated protein with a non-functional death domain. Cell lines transfected with these *FAS* mutations had impaired apoptosis compared to cells transfected with FAS WT, but they did not confer any resistance to chemotherapy (Figure 3)[62]. However, this *in vitro* model lacked the natural microenvironment that forms the basis of FAS-FASL interactions and a functional immune system. We therefore used an immune competent *in vivo* animal model for understanding the role of *FAS* mutations on lymphoma growth and chemo-sensitivity[62]. We used the *Eμ-Myc* lymphoma model given that it successfully recapitulated the aggressive phenotypes of P53 mutated and BCL2+ lymphomas[42,45]. We initially transduced an *Eμ-Myc/Arf-null* chemo sensitive lymphoma line with either *Fas*(Y224*) (murine equivalent of *FAS*(Y232*)), Fas WT or empty vector controls. Our pilot study supported our hypothesis, whereby *Fas* mutant lymphomas had a significantly higher growth rate than the empty vector controls and the Fas wild type lymphomas *in vivo* (Figure 4)[62]. While we used a chemo-sensitive cell line, the aggressivity of the Fas mutant lymphomas made it challenging to assess the effect of the chemotherapy because all mice died shortly after being treated. Furthermore, Fas WT controls were too challenging to generate because the additional copies of the *Fas* gene induced apoptosis, which resulted in a poor transduction efficiency and poor viability in our controls[62].

To circumvent the challenges faced with virally transducing *FAS* into lymphoma cell lines, we decided to breed *Eμ-Myc* mice with *lpr* mice to generate mice that have different *Fas* genotypes and that could develop spontaneous lymphomas. This model would have several potential advantages over using lymphoma cell lines. The primary lymphomas would have physiological

levels of Fas and Fas mutant (2 wild type Fas or one copy of each) providing a better Fas wt control given that extra copies of Fas may be pro-apoptotic and less aggressive. The immune response generated in C57BL/6 mice when transplanted with these primary lymphoma will solely be due to exposure to lymphoma antigens and not viral antigens introduced during the transduction procedure. Unlike cell lines, primary lymphoma cells do not proliferate spontaneously in culture, and thus may have a slower growth rate *in vivo*, giving us more time to evaluate the effect of chemotherapy. Finally, this model may better reflect the biological heterogeneity of E*μ-Myc*/Fas$^{wt/wt}$ and E*μ-Myc*/Fas$^{lpr/wt}$ lymphomas, as they may have a different clonal evolution pattern, activate different oncogenes (e.g. *Bcl2*) and silence different tumor suppressor genes *(Tp53).*

Hypothesis

We hypothesize that *FAS* mutations accelerate lymphoma growth and confer therapeutic resistance to conventional chemotherapy.

Objectives

- Generate and genotype mice from breeding *Eμ-Myc* mice with *lpr* mice
- Monitor lymphoma development and growth in mice according to genotype: E*μ-Myc*/Fas$^{wt/wt}$, E*μ-Myc*/Fas$^{lpr/wt}$ and Fas$^{lpr/wt}$ (heterozygous; one wt allele and one *lpr* allele) mice.
- Monitor lymphoma development, growth, and overall survival (OS) of mice with different *Fas* genotypes (E*μ-Myc*/Fas$^{lpr/wt}$ and E*μ-Myc*/Fas$^{wt/wt}$) in immune-competent C57BL/6 (second generation).

- Study the sensitivity/resistance to CHOP (doxorubicin, cyclophosphamide, and vincristine) by injecting secondary generation lymphomas (Fas$^{lpr/wt}$ and Fas$^{wt/wt}$) i.e.
  lymphomas that were recovered after being injected into C57BL/6 mice.

2.2 Methods

2.2.1 Animals and housing

Eμ-Myc, *lpr* mice and all the C57BL/6 mice were purchased from Charles River Laboratories. All the mice were housed in clean, autoclaved cages in the Animal Facility, Institute Lady Davis, Jewish General Hospital. 6-8 weeks old female mice were purchased and left to acclimatize for 7 days in 12-hour light-dark cycles before experiment.

2.2.2 Breeding, genotyping, and generation of primary lymphomas

The *Eμ-Myc* transgenic mouse is one of the best models to study *Myc* driven aggressive B cell lymphomas like DLBCL[42,45,46]. The tumors are typically represented morphologically by massive lymphadenopathy and splenomegaly. The *c-myc* gene is placed under the IgH enhancer which is established to develop spontaneous lymphomas in about 90-100% of the cases[45]**.** The *lpr* mice have dysfunctional *FAS* gene due to the insertion of early transposable element into intron 2 [88]**.** All the mice pups were genotyped using either ear/tail DNA sample by PCR. DNA was purified from the mice ear/tail tissue by using AllPrep Kit (QIAGEN). The primers used for amplification of IgHMyc: Transgene forward- 5 TTA GAC GTC AGG TGG CAC TT 3; Transgene reverse- 5 TGA GCA AAA ACA GGA AGG CA 3; Internal positive control forward- 5 CTA GGC CAC AGA ATT GAA AGA TCT 3; Internal positive control reverse- 5 GTA GGT GGA AAT TCT AGC ATC ATC C 3. The primers used for amplification of Fas^{lpr}: Common- 5 GTA AAT AAT TGT GCT TCG TCA G 3; Mutant- 5 TAG AAA GGT GCA CGG GTG TG 3; Wildtype- 5 CAA ATC TAG GCA TTA ACA GTG 3. All the primers were purchased from The Integrated DNA technologies. The PCR products were separated by gel electrophoresis in 2.5% agarose gel. The resulting E*μ-Myc* mice were either $Fas^{wt/wt}$ or $Fas^{lpr/wt}$ (mutated Fas allele). These mice were monitored for lymphoma development (primary E*μ-Myc*/Fas lymphoma generation ~ 3-6 months) and survival. When these mice reached a humane end (body condition score <2, paralysis) they were euthanized. Tissues (cervical and inguinal lymph node and spleen) were harvested for creating single cell suspensions (frozen immediately in FBS+10% DMSO freezing medium) and fixed in 500μL, 10% formalin solution (Anachemia Canada.Co) for later immunohistochemistry assay.

2.2.3 Assessment of lymphoma development and survival of Eμ-*Myc*/Fas$^{wt/wt}$ and Eμ-*Myc*/Fas$^{lpr/wt}$ primary lymphomas in C57BL/6 mice

The frozen primary Eμ-*Myc*/Fas lymphomas were thawed in RPMI and 20% FBS medium, washed once with phosphate buffered saline (PBS) and resuspended in PBS for injection. We determined the number of viable tumour B and T cells in the tumor cell suspension by flow cytometry. The cells were washed with PBS + 2% FBS and blocked with Fc block (BD Pharmingen 553142). It was then stained with anti CD19 (BV786 BD 563333, 1:1) and anti CD3 (PE Hamster BD 561824, 1:2) and analysed using BD LSR Fortessa Analyser (LDI Flow Cytometry facility). $1x10^6$ viable malignant B cells (re-suspended in PBS) of both Eμ-*Myc*/Fas$^{wt/wt}$ and Eμ-*Myc*/Fas$^{lpr/wt}$ primary lymphomas were injected into 6-8 weeks old female C57BL/6 mice by tail-vein intravenous injection. They were monitored for lymphoma progression and survival stratified according to *Fas* genotypes every 2 days initially then every day once lymphoma developed. Lymphoma development was monitored by measuring the tumor volume by non-invasive 3-D Ultrasound VEVO770 (Visualsonics) of both the right and left inguinal node and the cervical lymph node at regular time intervals. When these mice reached a humane end (Body condition score < 2, or paralysis) they were euthanized. The inguinal and the cervical lymph node and the spleen tissues were collected for creating single cell suspensions (frozen immediately) and an additional node was fixed in 500µL, 10% formalin solution and embedded in paraffin.

2.2.4 Determine the effects of Fas genotype on chemosensitivity

The frozen secondary Eμ-*Myc*/Fas lymphomas were thawed, analysed for viability, and injected into 6-8 weeks old C57BL/6 mice using the same methodology as 2.2.3. Once the tumor became palpable and reached a size of 15~20 mm^3, they were treated one time with the drugs such as doxorubicin, cyclophosphamide, and vincristine (Jewish General Hospital oncology department pharmacy) according to the weight of the mice. The drugs doxorubicin (10 mg/kg) and cyclophosphamide (150 mg/kg) were administered by intra-peritoneal (IP) injection while vincristine (1:10, 0.5mg/kg) was administered by tail-vein intravenous (IV) injection. The mice treated with these drugs were housed in separate cages in the quarantine room. The response to treatment was monitored by measuring the tumor volume for remission/relapse until humane end. A schematic representation of the methodology is represented below (Figure 2).

2.2.5 Complete blood count (CBC)

A whole blood count assay was performed for those C57BL/6 mice injected with secondary Eμ-*Myc*/Fas lymphomas that died within 9 days of injection (developed lymphoma too fast and died before treatment). 25~50 µL blood was taken from the tail-vein on day 5 post tumor injection (prior to therapy) and analysed in scil *Vet* ABC (Animal blood counter) in the animal quarters, Lady Davis Institute. The procedure was repeated on humane end just prior to euthanizing the mice (Figure 14).

2.2.6 Statistical Analysis

All the statistical analysis was performed in IBM SPSS statistics software version 11.0. The graphs and plots were generated both in Microsoft Excel and SPSS software. The time to lymphoma development of the two Fas genotypes in both primary and secondary generation and Kaplan-Meier Survival plots of the two Fas genotypes in primary, secondary and third generation were plotted and compared by the log-rank (Mantel–Cox) test. The tumor volume of the two groups in the secondary lymphoma generation was represented using Box and Whisker plots.

The statistical significance differences were analysed using unpaired Student's *t*-test. The level of significance was set at $p < 0.05$.

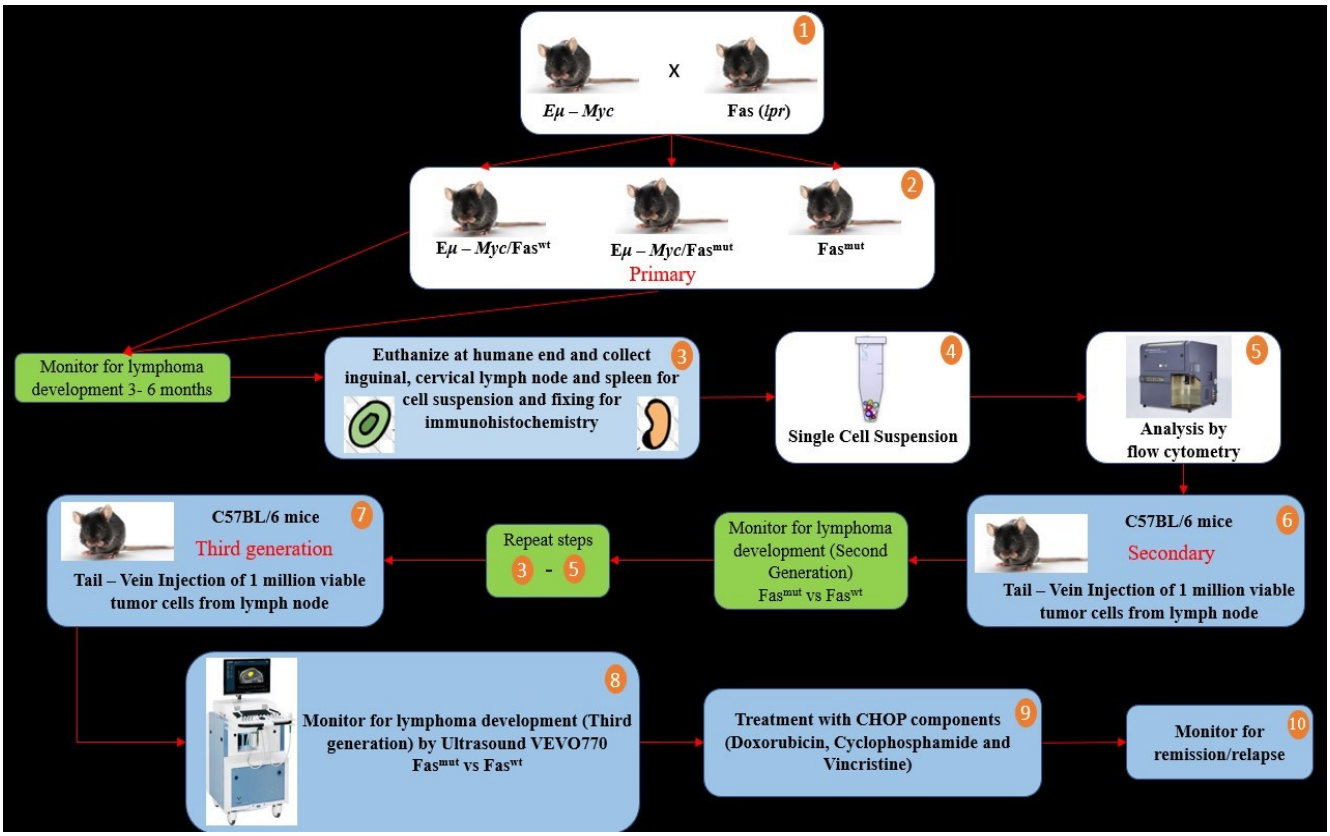

Figure 2. Schematic representation of methodology

2.3 Results

2.3.1 Different FAS genotype mice generated from breeding *Eμ-Myc* and *lpr* mice

142 pups generated from breeding were genotyped and sorted according to different Fas genotypes: 37 E*μ-Myc*/Fas$^{wt/wt}$, 17 E*μ-Myc*/Fas$^{lpr/wt}$, 20 Fas$^{lpr/wt}$ and 70 Fas$^{lpr/lpr}$. Of these, 21/37 E*μMyc*/Fas$^{wt/wt}$ (56.75%) and 10/17 (58.82%) E*μ-Myc*/Fas$^{lpr/wt}$ developed lymphoma (p = 0.563). Mice without the *Eμ-Myc* transgene (i,e Fas$^{lpr/wt}$ and Fas$^{lpr/lpr}$) did not develop lymphoma within 9 months of observation.

2.3.2 Time to lymphoma development is not different in both Fas$^{lpr/wt}$ and Fas$^{wt/wt}$ primary lymphomas

The primary E*μ-Myc*/Fas$^{wt/wt}$ and E*μ-Myc*/Fas$^{lpr/wt}$ mice take 3-6 months to spontaneously develop lymphoma. Our hypothesis from the results of previous studies in our lab states that *FAS* mutation accelerates lymphoma growth[62]. But there was no significant difference in time to lymphoma development observed in both Fas groups (Figure 5A). The median time to lymphoma development in the E*μ-Myc*/Fas$^{wt/wt}$ cohort was observed to be 161 days and the median time to lymphoma development in the E*μ-Myc*/Fas$^{lpr/wt}$ cohort was observed to be 164 days (p= 0.943).

The overall survival (number of days the mice survived before euthanizing, due to any of the following reasons: lymphoma/ sickness due to any other reasons/ hydrocephaly/ malocclusion/ opaque eyes) of E*μ-Myc*/Fas$^{wt/wt}$ and E*μ-Myc*/Fas$^{lpr/wt}$ mice generated from breeding *Eμ-Myc* and *lpr* mice was not different (Figure 5B). The Kaplan-Meier survival according to the genotype was not significantly different (p= 0.510).

2.3.3 Fas$^{lpr/wt}$ lymphomas engraft better into immune-competent C57BL/6 mice compared to Fas$^{wt/wt}$ lymphomas

To date, 28 E*μ-Myc* primary lymphomas (10 E*μ-Myc*/Fas$^{lpr/wt}$ and 18 E*μ-Myc*/Fas$^{wt/wt}$) have been injected into recipient immune-competent C57BL/6 mice (in duplicates except one Fas$^{wt/wt}$ lymphoma which was injected into only one recipient mice due to availability of just 1x10^6 cells). Of these, 4/10 E*μ-Myc*/Fas$^{lpr/wt}$ and 5/18 E*μ-Myc*/Fas$^{wt/wt}$ lymphomas, i.e. primary lymphomas, engrafted and grew in both recipient mice, 4/10 E*μ-Myc*/Fas$^{lpr/wt}$ and 3/18 E*μ-Myc*/Fas$^{wt/wt}$ lymphomas engrafted into only one of the two recipient mice. The remaining lymphomas, 2/10

E*μ-Myc*/Fas$^{lpr/wt}$ and 10/18 E*μ-Myc*/Fas$^{wt/wt}$ failed to generate lymphoma in at least 2 recipient C57BL/6 mice. Thus, of the primary lymphomas generated from breeding, the Fas mutant lymphomas had a slightly better engraftment rate in immune competent mice but it was not statistically significant (8/10 vs 8/18, p= 0.119).

2.3.4 The second generation E*μ-Myc*/Fas$^{lpr/wt}$ lymphomas grow faster than the E*μ-Myc*/Fas$^{wt/wt}$ lymphomas in the recipient immune-competent C57BL/6 mice

To study the tumor growth of E*μ-Myc*/Fas$^{lpr/wt}$ and E*μ-Myc*/Fas$^{wt/wt}$ primary lymphomas in immune-competent C57BL/6 mice, we injected a total of 55 recipient C57BL/6 mice with 28 primary E*μ-Myc* lymphomas. In total, 35/55 were injected with Fas$^{wt/wt}$ (in duplicates except one) and 20/55 were injected with Fas$^{lpr/wt}$ primary lymphomas (in duplicates). Out of these, 14/20 Fas$^{lpr/wt}$ and 12/35 Fas$^{wt/wt}$ developed lymphoma (p= 0.011).

The time to lymphoma development was shorter in C57BL/6 mice injected with Fas$^{lpr/wt}$ lymphomas compared to the mice injected with Fas$^{wt/wt}$ lymphomas (Figure 6A). The mean time to develop lymphoma was 49 days in the Fas$^{lpr/wt}$ cohort compared to 95 days in the Fas$^{wt/wt}$ cohort (p= 0.045). The time to lymphoma development is much shorter in the second generation (~1-2 months) compared to the primary spontaneous lymphoma (~3-6 months). However, the overall survival of mice injected with Fas$^{lpr/wt}$ and Fas$^{wt/wt}$ lymphomas was not different (Figure 6B). The median overall survival of mice injected with Fas$^{lpr/wt}$ lymphomas was 29 days and the median overall survival of mice injected with Fas$^{wt/wt}$ lymphomas was 32 days (p=0.642). There were 3 Fas$^{wt/wt}$ lymphomas that did not develop a tumor when injected in the recipient mice but all the mice that had been injected with those lymphomas were in the same cages and died on day 15, 16 and 21, for reasons unknown. These 3 lymphoma genotypes will be repeated for observation of tumor development and survival.

2.3.5 E*μ-Myc*/Fas$^{lpr/wt}$ secondary lymphomas were not larger than E*μ-Myc*/Fas$^{wt/wt}$ secondary lymphomas

The tumor development and progression were monitored through non-invasive 3-D Ultrasound VEVO770 (Visualsonics) by measuring tumor volume at regular time intervals.

Lymphadenopathy was observed in all the mice that developed lymphoma. The cervical and the inguinal lymph nodes were measured and tumor volume was calculated. The Eμ-*Myc*/Fas$^{lpr/wt}$ secondary lymphomas had an aggressive course, shorter time to lymphoma development but the maximal tumor volumes at the time of euthanasia were not significantly different compared to Eμ-*Myc*/Fas$^{wt/wt}$ secondary lymphomas, likely because tumor size was one of the defined endpoints for euthanization. The mean tumor volume in the Eμ-*Myc*/Fas$^{lpr/wt}$ cohort was 89.56 mm^3 and the mean tumor volume was 72.94 mm^3 in the Eμ-*Myc*/Fas$^{wt/wt}$ cohort (p=0.618) (Figure 13).

2.3.6 Effect of Fas genotype on chemosensitivity?

To study the resistance to chemotherapy, we injected Eμ-*Myc*/Fas$^{lpr/wt}$ and Eμ-*Myc*/Fas$^{wt/wt}$ secondary lymphomas, i.e. only the most aggressive primary lymphomas that were able to generate tumors, in C57BL/6 mice. There was no difference in lymphoma development in the recipient mice injected with secondary lymphomas (10 Eμ-*Myc*/Fas$^{lpr/wt}$ and 9 Eμ-*Myc*/Fas$^{wt/wt}$ secondary lymphomas were injected; p=0.292). Furthermore, survival of these C57BL/6 mice (untreated controls) injected with Eμ-*Myc*/Fas$^{lpr/wt}$ and Eμ-*Myc*/Fas$^{wt/wt}$ secondary lymphomas was not different in both the cohorts. The median overall survival was 20 days in the Fas$^{wt/wt}$ group while the median overall survival was 15 days in the Fas$^{lpr/wt}$ group (p= 0.181) (Figure 7). The Kaplan-Meier survival curve was generated for the control mice injected with the secondary Eμ-*Myc* lymphomas (Figure 8).

2.3.7 Doxorubicin and Cyclophosphamide have the best response, but relapse is a common phenomenon

There was a significant improvement in the overall survival for mice given chemotherapy compared to the untreated controls within the same genotype (p= 0.004 for both the groups) (Figure 8 A&B). Overall, doxorubicin provided the best tumor control, but in all cases, the lymphoma relapsed. In doxorubicin treated mice, the median OS of the Fas$^{lpr/wt}$ group (n= 12) was 24 days compared to 31 days in the Fas$^{wt/wt}$ group (n=14), but this was not statistically significant (p=0.22) (Figure 10). In cyclophosphamide treated mice, the median survival of the Fas mutant group was 27 days (n=9) compared to 38 days in the Fas$^{wt/wt}$ group (n=9), (p=0.42) (Figure 11) . In vincristine treated mice, the median OS showed a similar trend to being shorter in the Fas$^{lpr/wt}$ group as the median survival was 17 days (n=7) compared to 38 days in the Fas$^{wt/wt}$ group (n=6), p=0.140)

(Figure 12). In doxorubicin and cyclophosphamide treated mice, the duration of remission was shorter in the Eμ-*Myc*/Fas$^{lpr/wt}$ group (p=0.047 for doxorubicin and p=0.058 for cyclophosphamide treatments). Overall, the disease progression and survival were poor in the mutant cohort compared to the wildtype cohort. This did not reach statistical significance in vincristine treated mice.

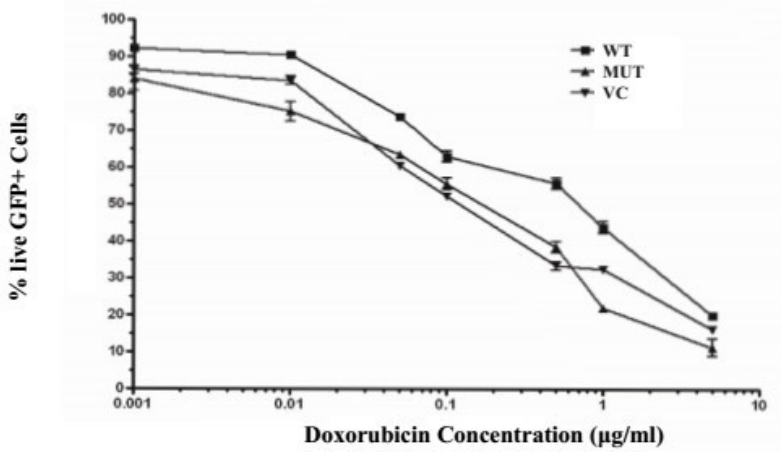

Figure 3. Cytotoxicity curve of doxorubicin on for Eμ-*Myc* cells. WT: FasWT; MUT: Fas(Y224*) mutant; VC: empty vector control[62]

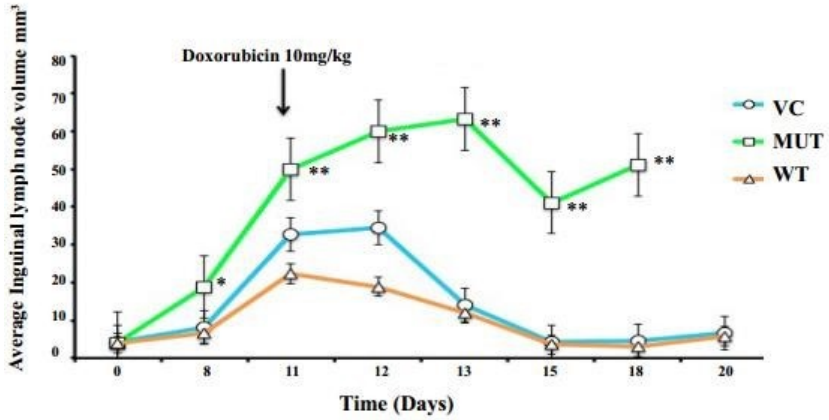

Figure 4. Fas genotype modulates lymphoma growth in immunocompetent mice; Lymph node volumes of mice transplanted with Eμ-*Myc* lymphomas of three different Fas genotypes- Fas(Y224*), Fas wt, empty Vector control[62]

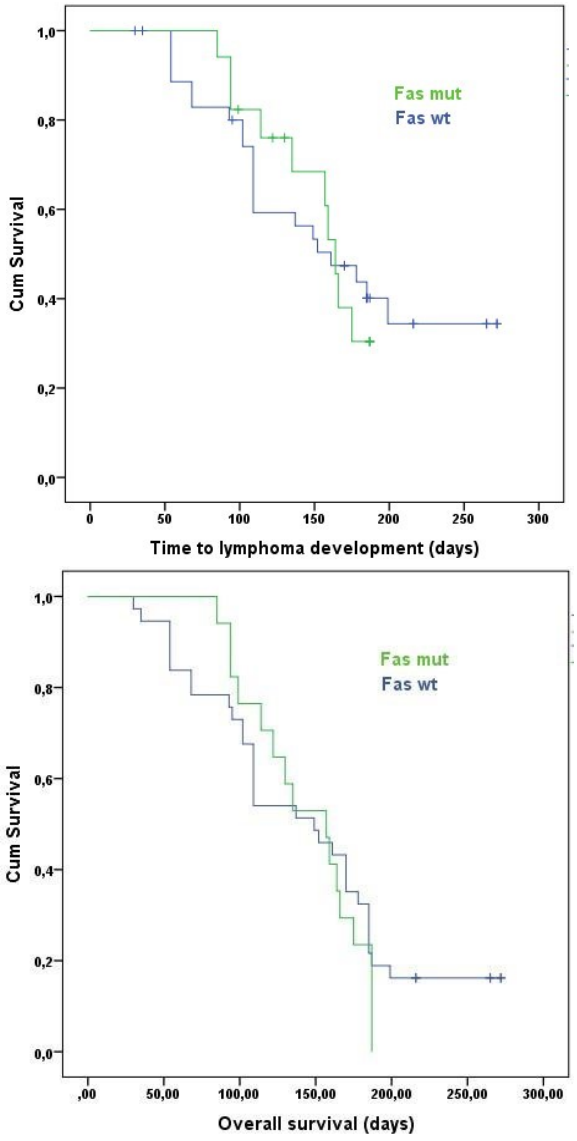

Figure 5. A) Time to lymphoma development in $Fas^{lpr/wt}$ vs $Fas^{wt/wt}$ primary generation, **p=0.943**; B) Overall survival of primary $Fas^{lpr/wt}$ vs $Fas^{wt/wt}$ lymphoma mice generated from breeding, **p=0.510.**

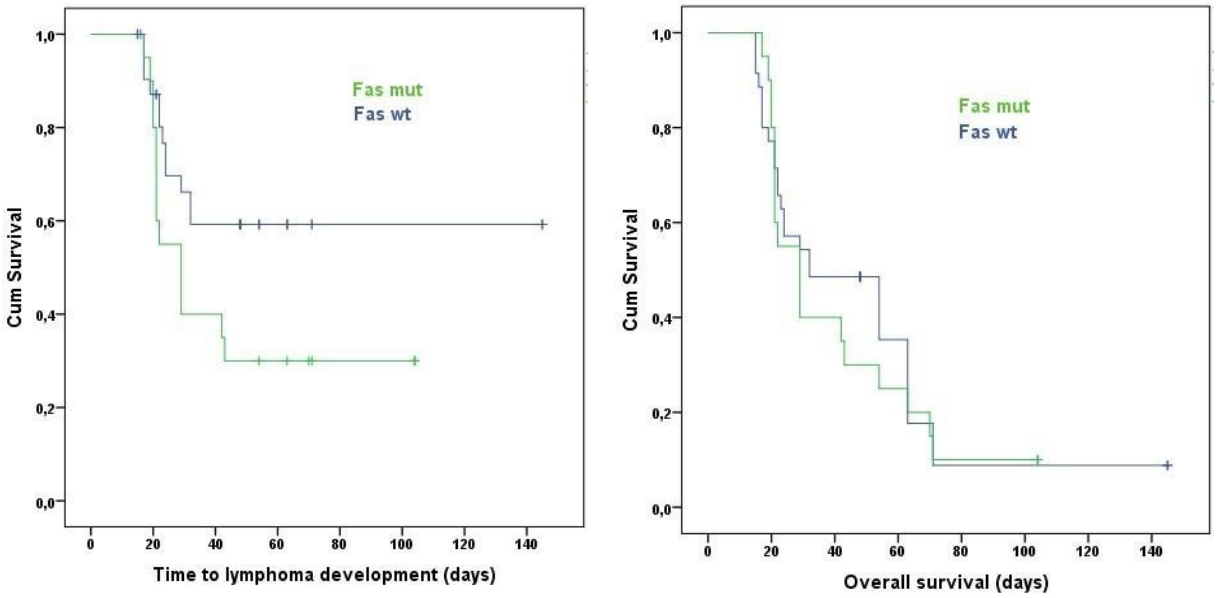

Figure 6. A) Time to lymphoma development in $Fas^{lpr/wt}$ vs $Fas^{wt/wt}$ second generation, **p=0.045**; B) Overall survival of second generation $Fas^{lpr/wt}$ vs $Fas^{wt/wt}$ lymphoma generated by injecting recipient C57BL/6 mice with primary lymphomas, **p=0.642**

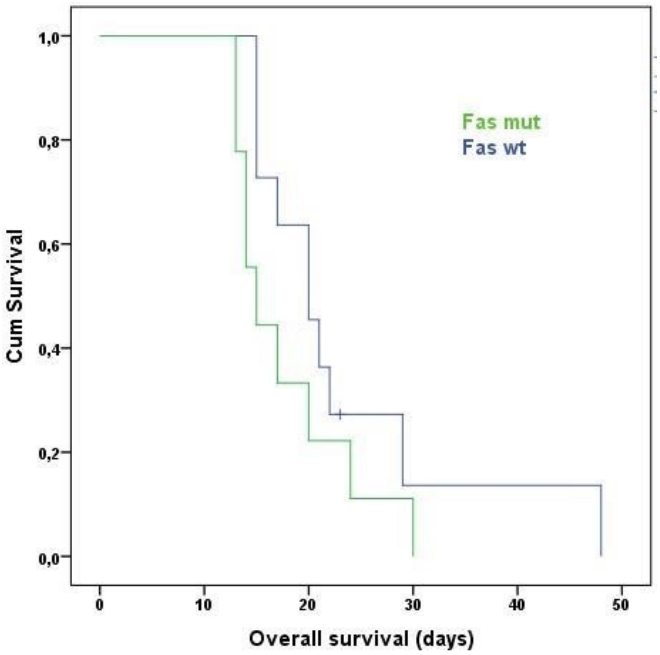

Figure 7. Overall survival of third generation $Fas^{lpr/wt}$ vs $Fas^{wt/wt}$ lymphomas generated by injecting recipient C57BL/6 mice with secondary lymphomas, **p=0.181**

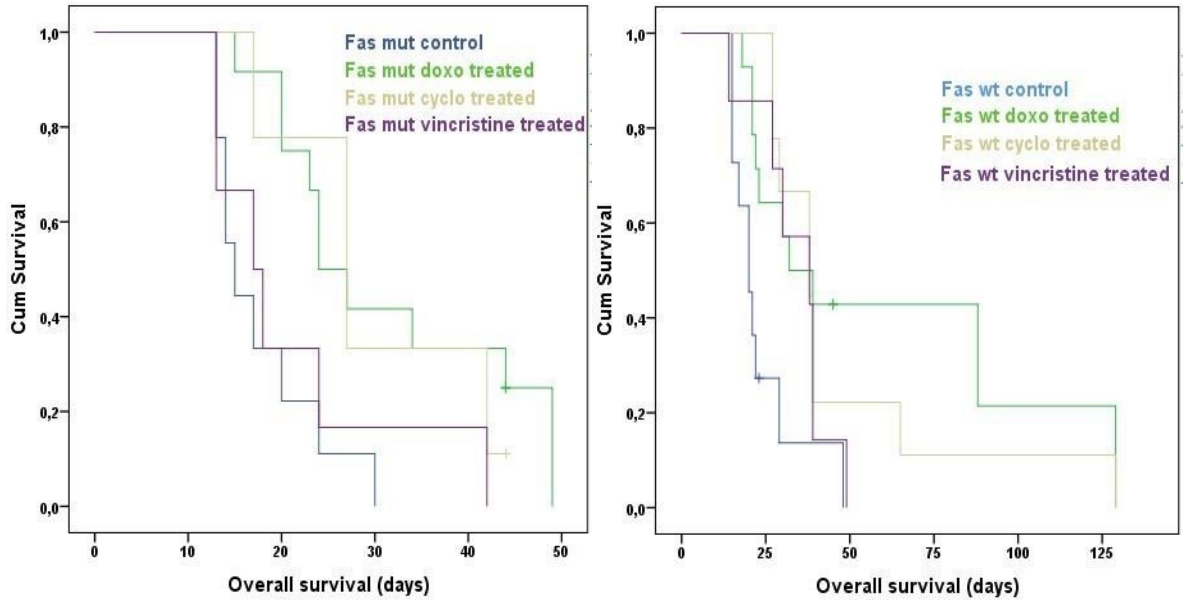

Figure 8. A) Overall survival of control and mice treated with doxorubicin, cyclophosphamide, and vincristine in the $Fas^{lpr/wt}$ third generation cohort, **p=0.004**; B) Overall survival of control and mice treated with doxorubicin, cyclophosphamide, and vincristine in the $Fas^{wt/wt}$ third generation cohort, **p=0.004**

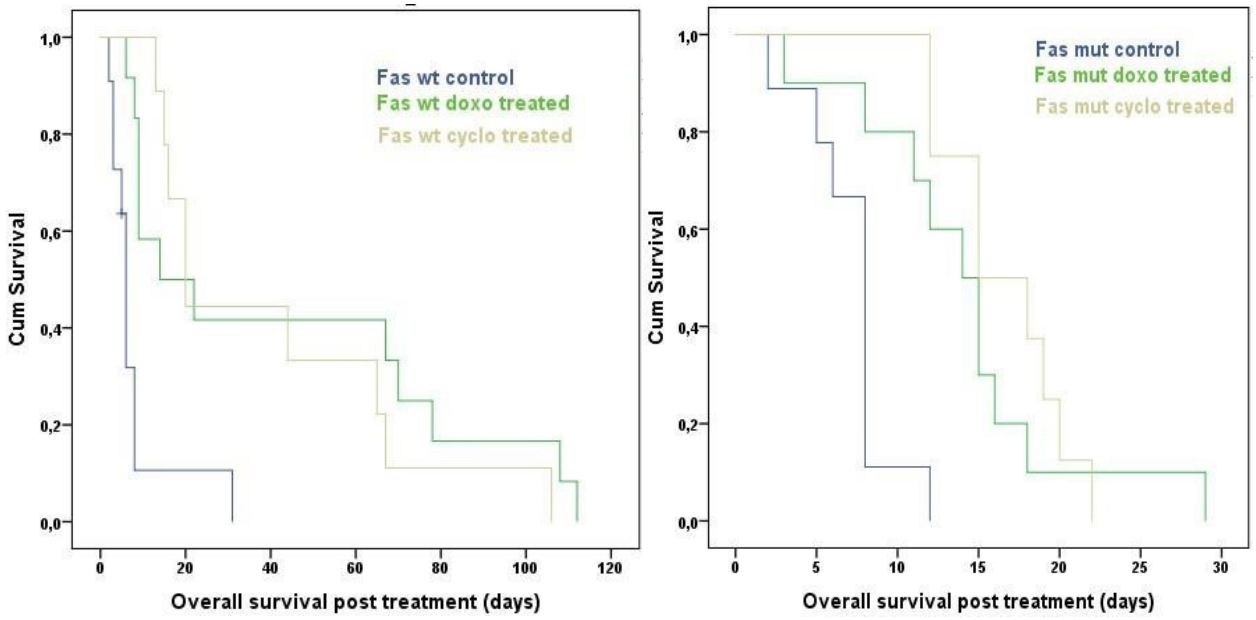

Figure 9. A) Overall survival post treatment of control and mice treated with doxorubicin and cyclophosphamide in the $Fas^{lpr/wt}$ third generation cohort, **p=0.000**; B) Overall survival post treatment of control and mice treated with doxorubicin and cyclophosphamide in the $Fas^{wt/wt}$ third generation cohort, **p=0.000**

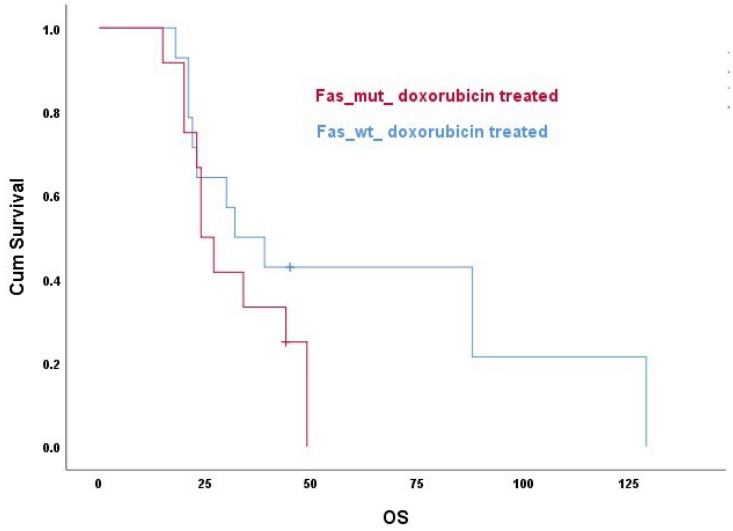

Figure 10. Kaplan Meier Overall survival for C57BL/6 mice injected with secondary Eμ-*Myc*/Fas$^{lpr/wt}$ lymphomas and Eμ-*Myc*/Fas$^{wt/wt}$ lymphomas and treated with A) Doxorubicin; **p=0.22**

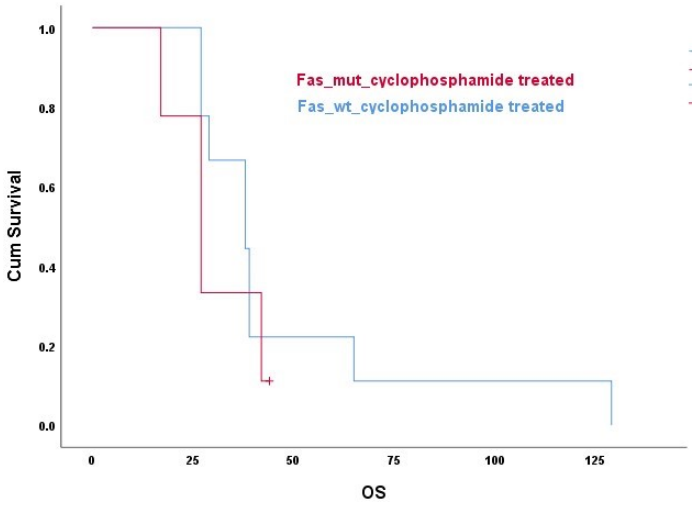

Figure 11. Kaplan Meier Overall survival for C57BL/6 mice injected with secondary Eμ-*Myc*/Fas$^{lpr/wt}$ lymphomas and Eμ-*Myc*/Fas$^{wt/wt}$ lymphomas and treated with B) Cyclophosphamide; **p=0.421**

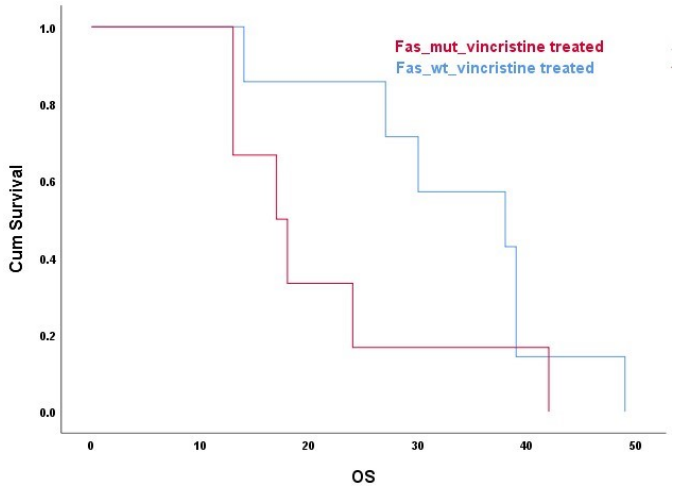

Figure 12. Kaplan Meier Overall survival for C57BL/6 mice injected with secondary Eμ-*Myc*/Fas$^{lpr/wt}$ lymphomas and Eμ-*Myc*/Fas$^{wt/wt}$ lymphomas and treated with C) Vincristine; **p=0.140.** Overall, no significance difference observed in both the cohorts.

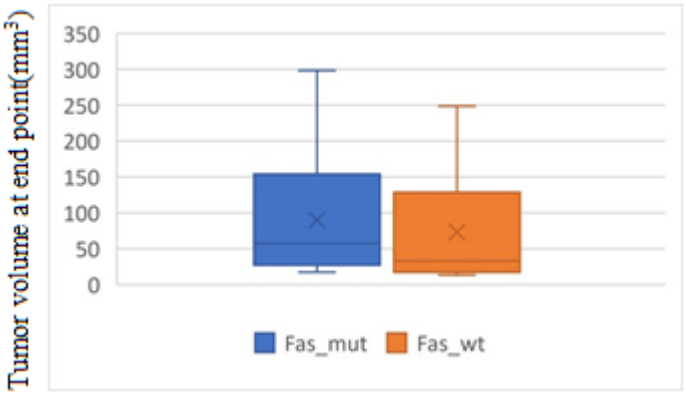

Figure 13. Tumor volume at end of Fas$^{lpr/wt}$ and Fas$^{wt/wt}$ lymphomas generated from injecting C57BL/6 mice with primary lymphomas, **p=0.61.**

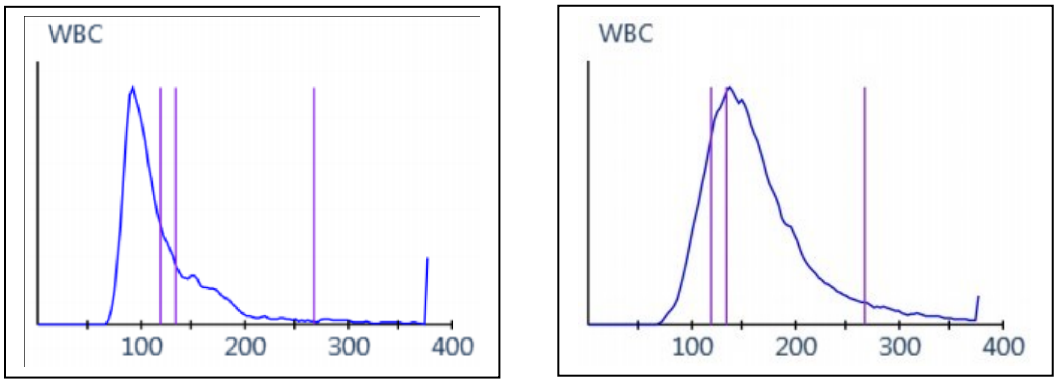

Figure 14. WBC count (complete blood count data) of an aggressive $Fas^{lpr/wt}$ lymphoma CTL on day 5 (7.97 $10^3/mm^3$ **normal**) and day 12 (55.28, after 1:3 dilution $10^3/mm^3$ **high)**

2.4 Discussion

We had identified recurrent *FAS* mutations in 15-20% of patients with rrDLBCL and had hypothesized that these mutations would accelerate lymphoma growth and confer resistance to standard chemotherapy[48,62]. The role of *FAS* mutations in lymphoma is unclear. The initial *in vitro* experiments carried out by our lab led to the observation that introduction of *FAS* mutations impaired apoptosis *in vitro* but did not confer resistance to chemotherapy. We postulated that the neighbouring immune cells in the microenvironment, which were absent *in vitro* (FAS-FASL interaction between neighbouring cells), were required for aggressive clinical course in *FAS* mutant lymphomas. Our initial data in cell lines demonstrated that *FAS* mutations accelerated lymphoma growth but this model was difficult to reproduce because introduction of FAS wild type controls would induce apoptosis in our cells[62]. To overcome this issue, we bred E*μ-Myc* and *lpr* mice to spontaneously generate Fas lymphomas of different genotypes in the E*μ-Myc* background. There was no difference in time to lymphoma development observed in the primary mice between E*μ-Myc*/Fas$^{lpr/wt}$ and E*μ-Myc*/Fas$^{wt/wt}$ groups. However, when these primary lymphomas were injected into C57BL/6 mice, E*μ-Myc*/Fas$^{lpr/wt}$ lymphomas engrafted better and grew faster compared to E*μ-Myc*/Fas$^{wt/wt}$ lymphomas. Furthermore, the duration of remission was significantly shorter in Fas$^{lpr/wt}$ but not all the lymphomas have been tested with chemotherapy (ongoing experiments). Taken together, our data with primary murine lymphomas supports that *FAS* mutations provide a growth advantage to lymphomas and may also participate in therapeutic resistance.

The *Eμ-Myc* transgene was the most important determinant of lymphoma development in primary mice, whereas the *Fas* genotype influenced growth only in established *Eμ-Myc* lymphomas transplanted in immune-competent mice. Our data suggests that the *Eμ-Myc* transgene is the determining critical event in lymphoma development in the primary generation of mice. The primary lymphoma generation is consistent with literature where B cell driven over-expression of MYC leads to lymphoma development[42]. The latency of lymphoma development was shorter in C57BL/6 mice (second generation) compared to spontaneous development in the primary generation. In contrast to what has been reported in the literature, none of the primary *lpr* mice (without *Eμ-Myc* transgene) developed lymphoma within 9 months of observation, 70 mice had *lpr/lpr* in the homozygous state[89]. The Fas genotype did not have an impact on lymphoma growth

once the lymphomas developed in the primary generation. *Fas* mutation only accelerated lymphoma growth in the second generation. One explanation for this discrepancy may be that the mice carrying the *lpr* genotype had dysfunctional Fas signalling in all the cells, including T cells and macrophages, which is not the case in the recipient C57BL/6 mice. Thus, differences in the immune micro-environment could be different in the primary generation of lymphomas and impact lymphoma growth patterns. Furthermore, there was greater heterogeneity in terms of growth patterns for the primary E*μ-Myc* lymphomas of both genotypes compared to *Eμ-Myc* cell lines used in our pilot study, which consistently develop lymphoma after 10-14 days[62]. Thus, there may be several underlying genetic events that may also affect lymphoma progression that were not yet addressed in our study. Overexpression of pro-apoptotic Myc leads to other genetic alterations such as inactivation of *Tp53*, mutations in *Myc*, overexpression of Bcl2, downregulation of Bim, etc[42]. These events could be different in the both Fas genotypes and could have influenced the heterogeneity of growth patterns that were present in the primary lymphoma cells compared to cell lines.

Our findings that *Fas* mutations accelerate lymphoma growth in immune competent mice support other work in the literature stating that the FAS/FASL signalling is important in cancer control. The initial *in vivo* experiments conducted by our lab stated that *FAS* mutations provide a growth advantage for lymphoma. Although *FAS* mutations have been reported in many haematological malignancies, its role in lymphoma biology is unclear. Additionally, our data validates the findings from our initial experiments, showing that primary E*μ-Myc*/Fas$^{lpr/wt}$ lymphomas grow faster than E*μ-Myc*/Fas$^{wt/wt}$ lymphomas when injected in C57BL/6 mice. Primary E*μ-Myc*/Fas$^{lpr/wt}$ lymphomas engrafted better (~ 1X10^6 cells engrafted) into C57BL/6 mice and had an aggressive phenotype, which was a significant trend compared to E*μ-Myc*/Fas$^{wt/wt}$ lymphomas. As mentioned already, the mean time to lymphoma development in Fas$^{lpr/wt}$ was 49 days compared to 95 days in Fas$^{wt/wt}$ lymphomas, significantly different($p=0.045$). The FAS/FASL pathway is the major system that CD8+ T cells deploy to eliminate virally infected or cancer cells apart from perforin/granzyme and another indirect cytokine mediated elimination[75]. Mutations in the primary structure of *FAS* might be a possible mechanism of immune surveillance evasion and support tumor growth[72]. Somatic mutations clustered in the DD of the FAS receptor promoted tumor growth in 43 cases of gastric cancer[73]. There is another study in non-lymphoid cancers, such as lung cancer where alterations in *FAS* gene might inhibit apoptosis and contribute to tumorigenesis

in small cell lung cancer[90]. Germline *FAS* mutations lead to autoimmune lymphoproliferative syndrome whereas somatic mutations have been associated with multiple cancers[11]. Somatic *FAS* mutations play a major role in lymphomagenesis of Non-Hodgkin lymphomas, suggesting a link between *FAS* mutations, cancer, and autoimmunity. A study by Afshar-Sterle et al. states that FAS-FASL interactions are critical in controlling lymphoma progression where deficiency of T cells accelerated the onset of B cell lymphoma in mice[68].

Our data suggests that *FAS* mutations may also be contributing to therapeutic resistance. The overall survival of mice treated with doxorubicin and cyclophosphamide was shorter in the $Fas^{lpr/wt}$ group compared to the $Fas^{wt/wt}$ group, with the biggest differences observed in the doxorubicin treated mice compared to untreated controls, although these were not statistically significant (Figure 9). Vincristine did not seem to have any effect on lymphoma compared to the other two drugs since the overall survival in both the groups were similar. The survival post treatment was shorter in the $Fas^{lpr/wt}$ cohort i.e. time to relapse was shorter compared to the $Fas^{wt/wt}$ cohort for both doxorubicin and cyclophosphamide treatments. Hence, *FAS* mutations might confer resistance to chemotherapy. However, this can be validated by treating more lymphomas in the future, as not all our primary lymphomas had been assessed for chemo-sensitivity at the time of the thesis presentation. Many studies have focused on the role of chemotherapy in engaging FAS-FASL interactions between the lymphoma cells and the neighbouring lymphocytes in the tumor microenvironment to initiate apoptosis. Our lab showed that Fas expression on malignant B cells increased both *in vitro* and *in vivo* after conventional chemotherapy suggesting that chemotherapy engages through the extrinsic apoptotic pathway[62]. This suggests that the tumor microenvironment plays a major role in chemotherapy induced cell death[62]. DNA damaging agents like anthracyclines (doxorubicin) are shown to enhance Fas expression[84]. The study by Muller et al. states that wildtype p53 is required for Fas mediated apoptosis post DNA damage through drugs but most of the tumors have mutant p53[84]. Up regulation of Fas receptor after DNA damage caused by post drug treatments seems to be p53 dependent[84]. Doxorubicin induced Fas mediated apoptosis in human thyroid carcinoma cells[91]. This certainly highlights the importance of investigating the role of *FAS* mutations in therapeutic resistance.

In summary, our data demonstrate that *FAS* mutations provide a growth advantage and contribute to tumorigenesis in DLBCL. The duration of remission was shorter in the $Fas^{lpr/wt}$ group compared to the $Fas^{wt/wt}$ group when mice were treated with doxorubicin and cyclophosphamide,

supporting that *Fas* mutations might also confer resistance to chemotherapy. There is great heterogeneity observed within and across Fas genotypes. Understanding the complex tumor biology of DLBCL and the oncogenic pathways to lymphoma development is the key to unravelling therapeutic resistance. The genomic alterations that evolve in these lymphomas will be determined to see if there are any differences in the mutation profiles between the 2 Fas genotypes, but this was beyond the scope of this thesis project. Studying the interactions in the tumor microenvironment will also be an important step in understanding the anti-tumor responses[36]**.** Histological analysis of the lymphomas generated in this project is also underway, looking at different components of the immune response (different T cell subsets, B cells and monocytes) as well as expression of specific immune check point targets (e.g. PD1 and PDL1). Understanding the tumor biology and the microenvironment in these $Fas^{lpr/wt}$ and $Fas^{wt/wt}$ lymphomas might allow us to identify novel targets for more rationale combinations of therapies which could be effective in treating future patients afflicted with these aggressive lymphomas.

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Chapter 3: Conclusion and Summary

We studied the role of *FAS* mutations in lymphoma growth and therapeutic resistance. From the results of previous experiments conducted by our lab, we preferred to use the *in vivo* E*μ*-*Myc* model to generate spontaneous lymphomas by breeding *Eμ-Myc* and *lpr* mice because FAS requires activation from its ligand FASL from the neighbouring cells, which was absent *in vitro*. We generated primary E*μ*-*Myc*/Fas$^{wt/wt}$, E*μ*-*Myc*/Fas$^{lpr/wt}$ and Fas$^{lpr/wt}$ lymphomas (C57BL/6 mice background) and monitored them for lymphoma development. The onset of lymphoma took from 3~6 months in these mice and Fas$^{lpr/wt}$ without the *Eμ-Myc* did not develop lymphoma in the 9 months of observation. There was no difference in time to lymphoma development observed in both these groups. These primary E*μ*-*Myc* lymphomas were injected into recipient C57BL/6 mice to study the lymphoma growth and survival. An interesting observation was that primary Fas$^{lpr/wt}$ engrafted better into recipient mice compared to the Fas$^{wt/wt}$ lymphomas. Furthermore, C57BL/6 injected with primary Fas$^{lpr/wt}$ lymphomas developed lymphoma faster compared to mice injected with primary Fas$^{wt/wt}$ lymphomas. The latency of lymphoma development was shorter in the second generation compared to the primary generation (~1 month vs 3~6 months respectively). *FAS* mutations favoured lymphoma growth in the second generation in C57BL/6 mice but failed to do so in the primary generation.

To study the response to chemotherapy, secondary E*μ*-*Myc*/Fas$^{lpr/wt}$ and E*μ*-*Myc*/Fas$^{wt/wt}$ lymphomas injected into C57BL/6 mice were treated with components of CHOP, such as doxorubicin, cyclophosphamide, and vincristine. Doxorubicin and cyclophosphamide seemed to have a better response compared to vincristine, although relapse was a commonly observed phenomenon. The disease progression and survival post treatment were poor in the Fas$^{lpr/wt}$ cohort compared to the Fas$^{wt/wt}$ cohort for both doxorubicin and cyclophosphamide but mice in both the cohorts responded to therapy initially. We can conclude that *FAS* mutations accelerate lymphoma growth and might confer resistance to chemotherapy.

Heterogeneity is observed in lymphoma growth within the same *Fas* genotypes. This could be related to factors not yet measured in this project, such as the status of p53, overexpression of Bcl2, increased NF-κB signalling and other downstream signalling or any other genetic aberrations. Therapeutic resistance is a major problem in relapsed DLBCL patients.

Understanding the FAS/FASL interactions between FAS mutant lymphomas and cells within the tumor microenvironment may help identify novel therapies for treating aggressive lymphomas.

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