Recurrence 11q24.3 gain contributes to the pathogenesis of diffuse large B cell lymphoma by deregulating ETS1 and FLI1

TESTONI, Monica

Abstract

DLBCL is the most frequent and aggressive form of non-Hodgkin's lymphoma. The still high percentage of mortality is in part due to the heterogeneity of DLBCL at genetic level. Among genetic alterations, a recurrent gain at 11q24.3 was detected in 26% of DLBCL. It was associated with higher expression of ETS1 and FLI1 transcription factors. Silencing of both genes led to cell death and a block in proliferation in a DLBCL cell line bearing the specific 11q gain but only FLI1 silencing gave a similar phenotype in cell lines without the gain. At molecular level, ETS1 directly and negatively regulates BLIMP1 expression. Importantly, ETS1 or FLI1 silencing leads to changes in the expression of B cell important genes. This project reveals ETS1 and FLI1 as interesting drug targets to be further analyzed. In addition, the status of MYC gene was analyzed in this cohort of DLBCL patients treated with R-CHOP.
RECURRENT 11q24.3 GAIN CONTRIBUTES TO THE PATHOGENESIS OF DIFFUSE LARGE B CELL LYMPHOMA BY DEREGULATING ETS1 AND FLI1

THÈSE
présentée à la Faculté des sciences de l’Université de Genève pour obtenir le grade de Docteure ès sciences, mention sciences pharmaceutiques

par

MONICA TESTONI

dee
COMO (ITALY)

Thèse N° 4669
BELLINZONA 2014
Doctorat ès sciences
Mention sciences pharmaceutiques

Thèse de Madame Monica TESTONI

intitulée :

"Recurrent 11q24.3 Gain Contributes to the Pathogenesis of Diffuse Large B Cell Lymphoma by Deregulating ETS1 and FLI1"

La Faculté des sciences, sur le préavis de Monsieur Leonardo SCAPOZZA, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), Monsieur Francesco BERTONI, docteur et codirecteur de thèse (Oncology Institute of Southern Switzerland, Bellinzona, Switzerland), Monsieur Gérard HOPFGARTNER, professeur ordinaire (Section des sciences pharmaceutiques), Monsieur Pier Paolo PICCALUGA, professeur (Department of Experimental, Diagnostic, and Specialty Medicine Bologna, Unit of Hematopathology, University Medical School, Bologna, Italia) et Madame Margot THOME-MIAZZA, professeure (Département de biochimie, Université de Lausanne, Epalinges, Suisse), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 13 mai 2014

Thèse - 4669 -

Le Doyen, Jean-Marc TRISCONE

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".
Dedicato a tutti i miei nonni compreso don Titino...
a Nico e a tutti coloro a cui voglio bene..
e infine dedicato a chi presto arriverà...
“The man who is blind to the beauties of Nature has missed half the pleasure of life.”

-Lord Robert Baden Powell-
Diffuse large B cell lymphoma (DLBCL) is the most frequent B cell-derived lymphoma in adults accounting for 35-40% of all cases. The diversity in clinical presentation, as well as the pathologic and biologic heterogeneity, suggests that DLBCL comprises several disease entities. Gene expression profiling studies have identified at least three molecular subtypes of DLBCL: germinal center B cell-like (GCB) DLBCL, activated B cell-like (ABC) DLBCL, and primary mediastinal B cell lymphoma (PMBL). Each of these molecular subgroup is associated to a particular stage of B cell differentiation through plasma cell, although is not still clear the extent to which these subtypes maintain the molecular and physiological properties of their normal B cell counterparts. Moreover, the three DLBCL groups differ also in terms of pathology, with a large and diverse set of chromosomal copy number aberrations associated primarily or exclusively with specific DLBCL subtypes, and clinical behavior, with the worst overall survival (OS) for the ABC-DLBCL subgroup.

DLBCL is a very aggressive lymphoma and a large percentage of DLBCL patients are not fully curable yet with the current standard immuno-chemotherapy regimens (R-CHOP). Therefore, genomic and molecular analyses can identify crucial mechanisms of pathogenesis and new compounds to improve the efficiency of the therapeutic modalities. Normal germinal center (GC) B cell differentiation requires a complex transcriptional program and alterations of genes involved in this process are relevant for DLBCL pathogenesis.

Genomic DNA profiles of 166 DLBCL patients samples have been previously obtained in my group of research allowing the identification of genomic aberrations that are associated with this disease. Among these genetic aberrations there was gain that affected the 11q24.3 chromosome locus. This genomic alteration was identified in the 23% of our cohort of DLBCL patient and it was associated with higher expression of ETS1 (p=0.0129) and FLI1 (p=0.0082) genes both belonging to the ETS family of transcription factors. ETS1 and FLI1 closely map inside the 11q24.3 genomic region and were found concomitantly expressed in DLBCL patient samples. In order to assess whether this genomic alteration is associated to one of the GCB- or ABC-DLBCL subtypes, genomic analysis was integrated with the cell of origin data for each patients. No significant association were found. In contrast, ETS1 mRNA levels were higher in ABC-DLBCL subtype suggesting a specific oncogenic role of this gene in ABC-DLBCL lymphomagenesis.
Thus, being the two genes, ETS1 and FLI1, correlated in term of genomic mapping and mRNA expression in DLBCL patients, the aim of this project was to functional characterize these two genes as possible deregulators of late stages of B cell maturation in DLBCL. A first screening of 22 DLBCL cell lines was performed to evaluate ETS1 and FLI1 protein levels. DLBCL cell lines encompassed GCB- and ABC-DLBCL subtypes and most of them expressed both factors, albeit at different levels. By combining these data with genome-wide DNA profiling, the DLBCL cell line, OCI-LY7 was revealed as the suitable model to in vitro study the pathogenetic roles of ETS1 and FLI1 in DLBCL. In fact, OCI-LY7 DLBCL cell line presented both the 11q24.3 gain observed in clinical specimens and the highest protein levels of ETS1 and FLI1.

The approach was to silence the two ETS factors through lentiviral shRNA, utilizing 2 different hairpins for each gene and one hairpin against GFP as control. The reduction in their expression was confirmed by quantitative-RT-PCR and western blot analysis. Phenotypic effects in OCI-LY7 after ETS1 and FLI1 silencing were assessed showing that both ETS1 and FLI1 down-regulation induced a lower proliferation rate compared with the shGFP control. This phenotype was confirmed by an EdU in-vitro assay in which a reduced percentage of cells in S-phase was obtained in ETS1 or FLI1 silenced samples in respect to the control sample. Through a co-culture experiment, in which an equal amount of cells infected with a vector carrying the GFP marker (GFP positive) and with a vector carrying the shRNA against each ETS1 and FLI1 or GFP genes (GFP negative), the biological effects of ETS1 and FLI1 down-regulation was evaluated at longer time points. This experiment revealed that OCI-LY7 cells with reduced levels of ETS1 or FLI1 proliferate less efficiently. In fact, GFP positive cells outgrew OCI-LY7 GFP negative cells with the exception of cells infected with the shRNA against GFP, indicating an impairment of cell proliferation as a result of the ETS1 or FLI1 down-regulation. This phenotype was associated with cell death in OCI-LY7 indicating that these two genes are critical also for cell viability in OCI-Y7 DLBCL cell line.

To investigate whether ETS1 and FLI1 could cooperate in regulating cell viability in patients carrying the 11q24.3 gain, a concomitant down-regulation of ETS1 and FLI1 was performed in OCI-LY7. It was observed that the concomitant silencing of the two ETS factors clearly induced a stronger apoptosis compared to the individual silencing of these genes. This experiment strongly indicated that ETS1 and FLI1 could cooperatively sustain cell viability when both are deregulated in B cells, as it is the case of OCI-LY7 DLBCL cell line. In order to assess whether ETS1 and FLI1 are required for cell viability in primary DLBCL independently of the presence of the 11q24.3 gain and high levels of ETS1 and FLI1 expression, the viability of GCB- and ABC-DLBCL
derived cells lacking the 11q24.3 gain was evaluated after the single silencing of ETS1 and FLI1 genes. These analyses showed that only the FLI1 down-regulation significantly affected cell viability in a way similar to what was observed in OCI-LY7 cell line. In contrary, ETS1 down-regulation did not induce apoptosis in the other DLBCL cell lines. Interestingly the proliferation of these cell lines is affected by both ETS1 and FLI1 knock-down, suggesting a different role of the two genes in DLBCL models. Notably, ETS1 down-regulation in these cell lines cause the up-regulation of FLI1 expression that maybe compensate for the lost of ETS1 expression.

At molecular level, the expression of several important players in GC differentiation is affected following knocking-down of ETS1 and FLI1. The genes evaluated for the expression were: BCL2, BCL6, PAX5, IRF4, PRDM1, and XBP1. In particular, ETS1 down-regulation resulted in a significant up-regulation of PRDM1, the master regulator of PC differentiation, and this effect was followed by XBP1 up-regulation, whereas no effect on IRF4 was observed. These data were also confirmed at the protein level. Considering that all these factors are down-stream to NF-kB and IRF4 is expressed before PRDM1 gene, these data suggests that ETS1 might act up-stream or independently from the NF-kB pathway. To reinforce these data a rescue experiment was performed in OCI-LY7 cells previously infected with shETS1. This experiment showed that ETS1 reintroduction restored PRDM1 mRNA levels to values similar to the control cells (shGFP). As ETS binding site (EBS) were found within PRDM1 promoter, a chromatin immunoprecipitation (ChIP) analysis was performed by using antibody against ETS1 protein in OCI-LY7 interfered for the expression ETS1 or the control GFP. An enrichment of PRDM1 promoter region was detected in OCI-LY7 control sample, in which ETS1 level remain high because of the gain, while a reduced enrichment of PRDM1 promoter region was observed in OCI-LY7 interfered for ETS1 expression. Taken together, these data demonstrate that ETS1 directly and negatively regulates PRDM1 transcription in OCI-LY7. Interestingly, the same effect on PRDM1 expression is not observed in other DLBCL cell lines lacking the gain and this is due to genomic alterations of PRDM1 gene or in the PRDM1-negative regulator gene BCL6 that affect all DLBCL cell lines analyzed. Gene expression profiling analysis after ETS1 or FLI1 silencing in OCI-LY7 and other two DLBCL cell lines, SUDHL4 and SUDHL2, representative GCB- and ABC-DLBCL subtype respectively, showed changes in the expression of several genes in all the cell lines, but only in OCI-LY7 expression changes affect genes involved in cell cycle regulation, BCR signaling, plasma cell differentiation and chemotaxis. In particular, ETS1 had a positive effect on the expression of chemokine receptor CXCR5 demonstrated also by a ChIP analysis, while
FLI1 influence positively CXCR4 and CXCR7. All these chemokine receptors are important players during GC formation and development.

As conclusion, the gain at 11q24.3 determines a high expression of ETS1 and FLI1 that might contribute to the neoplastic phenotype by sustaining cell growth and deregulating the normal GC transcriptional program. ETS1 would mainly act by blocking the late stages of B cell differentiation thought direct suppression of PRDM1 expression and FLI1 act as a pro-survival factor. However, both ETS1 and FLI1 appear to regulate expression of genes important for the interaction of B cells with GC microenvironment reinforcing their important role in DLBCL lymphomagenesis.

Following the publication of these data in which FLI1 genetic silencing was demonstrated to be toxic for all DLBCL cell lines, we had the opportunity to start a collaboration with the group of research directed to Jeffrey Toretsky (Georgetown University, Lombardi Comprehensive Cancer Center, Washington DC, USA). Toretsky sent us the molecular compound YK-4-279, a small molecule that inhibits the activity of EWS-FLI1 fusion protein in Ewing’s Sarcoma, to evaluate its effect on DLBCL cell lines. The first analyses showed that YK-4-279 had anti-tumor activity in all the tested DLBCL cell lines.

Finally, this thesis had a secondary aim. Since genomic analysis is important for the prognosis of DLBCL patients and for the choice of therapy to be used, and because the clinical implication of MYC copy number gain was not clear yet, an analysis of MYC gene status on the cohort of 166 DLBCL patients treated with R-CHOP was performed. This analysis did not reveal any correlation between MYC copy number gain and worse OS of DLBCL patients.
Résumé

Le lymphome à grandes cellules B diffus (Diffuse Large B cell Lymphoma, DLBCL) est le lymphome le plus fréquent chez les adultes et représente 35 à 40% de la totalité des cas. La diversité clinique ainsi que l’hétérogénéité pathologique et biologique suggèrent que les DLBCL comprennent plusieurs entités pathologiques. Les études de profilage d’expression des gènes ont identifié au moins trois sous-types moléculaires de DLBCL: cellules B du centre germinatif (germinal center B cell-like) GCB-DLBCL, cellules B activés (activated B cell-like) ABC-DLBCL et le lymphome primaire de cellules B du médiastin (primary mediastinal B cell lymphoma, PMBL). Les grandes signatures génétiques qui distinguent ces sous-types indiquent qu’elles pourraient provenir de cellules B à divers stades de différenciation quoiqu’il ne soit encore pas clair jusqu’à quel point sont maintenues les caractéristiques moléculaires et physiologiques de leur équivalents sains. De plus, les trois groupes DLBCL diffèrent également en termes de pathologie, avec des aberrations conséquentes et diverses du nombre de copies chromosomiques, associées principalement ou exclusivement à des sous-types spécifiques de DLBCL et au comportement clinique, avec la pire survie globale (overall survival, OS) pour le sous-groupe ABC-DLBCL.

Le DLBCL est un lymphome très agressif et un grand pourcentage de patients ne répondent pas aux protocoles standards actuels d’immuno-chimiothérapie (R-CHOP). Dès lors, les analyses génomiques et moléculaires peuvent identifier des mécanismes cruciaux de la pathogénie et suggérer de nouvelles molécules pour améliorer l’efficacité des modalités thérapeutiques. La différenciation normale des cellules B du centre germinatif nécessite un programme transcriptionnel complexe et les altérations des gènes impliqués dans ce processus sont pertinentes pour la pathogenèse du DLBCL.

Les profils d’ADN génomique de 166 patients DLCBL ont permis à mon groupe de recherche l’identification d’aberrations génomiques associées à cette maladie, dont notamment un gain affectant le locus chromosomique 11q24.3. Cette altération génomique a été détectée dans 23% des patients de notre cohorte DLBCL et a été associée à une expression accrue des gènes ETS1 (p=0,0129) et FLI1 (p=0,0082) appartenant à la famille des facteurs de transcription ETS. ETS1 et FLI1 sont proches à l’intérieur de la région génomique 11q24.3 et sont exprimés de façon concomitante dans les échantillons de patients DLCBL. Afin de caractériser cette altération génomique comme associée à un des sous-types GCB- ou ABC-DLBCL, l’analyse génomique a été intégrée aux résultats des cellules d’origine pour chaque patient.
mais aucune association significative n’a été documentée. L’ARN messager de ETS1 était plus exprimé dans les sous-types ABC-DLBCL suggérant un rôle oncogénique pour ce gène dans la genèse des lymphomes ABC-DLBCL. Donc, avec ces deux gènes, ETS1 et FLI1, corréles en termes de « mapping » génomique et d’expression d’ARN messager chez les patients, le but de ce projet était de caractériser ces deux gènes comme dérégulateurs potentiels des stades terminaux de la maturation des cellules B dans les DLBCL. Un premier criblage de 22 lignées cellulaires DLBCL a été effectué pour évaluer le niveau d’expression de ETS1 et FLI1 au niveau protéique. Les lignées cellulaires DLBCL englobaient les sous-types GCB et ABC-DLBCL et la plupart se sont avérés exprimer les 2 facteurs, bien qu’à différents niveaux d’expression. En combinant ces résultats avec un profil d’ADN « genome-wide », la lignée cellulaire, OCI-LY7 s’est révélée comme le modèle de choix pour étudier in vitro les rôles pathogènes de ETS1 et FLI1 dans les DLBCL. En fait, OCI-LY7 présentait les deux gains 11q24.3 observés dans les spécimens cliniques et le plus haut niveau d’expression de ETS1 et FLI1. Une approche de silencing des deux facteurs de transcription au moyen de shRNA lentiviraux a été choisie, utilisant 2 hairpins différents pour chaque gène et un hairpin contre GFP comme contrôle. La baisse d’expression a été confirmée par une analyse PCR quantitative et par western blot. L’impact phénométique de la baisse d’expression de ETS1 et FLI1 sur OCI-LY7 a été évalué et a montré qu’une expression basse aussi bien de ETS1 que de FLI1 entraînait un taux de prolifération réduit comparé au contrôle shGFP. Ce phénotype a été confirmé par un essai EdU in vitro dans lequel il a été observée un pourcentage réduit de cellules en phase S du cycle cellulaire pour les échantillons avec silencing de ETS1 et FLI1 au regard de l’échantillon contrôle. Au travers d’une expérience de co-culture dans laquelle un nombre équivalent de cellules transfectées avec un vecteur GFP (GFP positif) et avec le shRNA pour les gènes ETS1, FLI1 ou GFP (GFP négatif), les effets d’une expression basse de ETS1 et FLI1 a été évaluée à plus long terme. Cette expérience a montré que les cellules avec le shRNA pour ETS1 et FLI1 proliféraient moins efficacement. En fait, les cellules GFP positives ont proliféré beaucoup plus que les cellules OCI-LY7 GFP négatives à l’exception des cellules contenant le shRNA pour la GFP, indiquant une détérioration de la prolifération cellulaire comme résultat de la régulation à la baisse de ETS1 et FLI1. Ce phénotype a également été associé avec la mort cellulaire des cellules OCI-LY7 suggérant que ces deux gènes sont également cruciaux pour la viabilité cellulaire de la lignée OCI-LY7 DLBCL.
Pour étudier si ETS1 et FLI1 pouvaient coopérer à la régulation de la viabilité cellulaire chez les patients porteurs du gain 11q24.3, une down-régulation concomitante des deux gènes a été opérée sur OCI-LY7. Nous avons observé que le silencing concomitant des deux facteurs ETS induisait clairement une augmentation de l’apoptose lorsque comparé au silencing d’un seul facteur. Cette expérience a fortement suggéré que ETS1 et FLI1 peuvent soutenir de manière coopérative la viabilité cellulaire lorsqu’ils sont tous les deux dérégulés dans les cellules B, comme dans le cas de la lignée OCI-LY7 DLBCL. Pour estimer si ETS1 et FLI1 sont requis pour la viabilité cellulaire dans les DLBCL primaires indépendamment du gain 11q24.3 et de leurs hauts niveaux d’expression, la viabilité de cellules dérivées de GCB- et ABL-DLBCL sans gain 11q24.3 a été évaluée lors du silencing de l’un ou de l’autre des deux gènes ETS1 et FLI1. Ces analyses ont montré que seule la down-régulation de FLI1 impactait significativement la viabilité de façon similaire à celle observée dans la lignée OCI-LY7. Au contraire, la down-régulation de ETS1 n’a pas induit d’apoptose dans ces autres lignées DLBCL. Il est toutefois intéressant de noter que la prolifération de ces lignées cellulaires est affectée par la down-régulation des deux facteurs ETS1 et FLI1 suggérant ainsi différents rôles pour ces deux gènes dans différents modèles de DLBCL. Notamment, la down-régulation des ETS1 dans ces lignées cellulaires entraîne une augmentation de l’expression de FLI1 qui pourrait compenser la perte d’expression de ETS1.

Au niveau moléculaire, l’expression de facteurs importants de la différentiation du centre germinatif a été affectée lors du silencing de ETS1 et FLI1. Nous avons étudié l’expression des gènes suivants : BCL2, BCL6, PAX5, IRF4, PRDM1 et XBP1. En particulier, la down-régulation de ETS1 a résulté dans une augmentation significative de l’expression de PRDM1, le principal régulateur de la différenciation en cellules plasmatiques. Cet effet a été suivi de l’augmentation de XBP1 alors que nous n’avons pas noté de différences dans l’expression de IRF4. Ces résultats ont été confirmés au niveau de l’expression des protéines. Considérant que tous ces facteurs sont en aval de NF-κB et que IRF4 est exprimé avant PRDM1, ces résultats suggèrent que ETS1 agit en amont ou indépendamment du pathway de NF-κB. Pour renforcer ces résultats, nous avons mis au point une expérience au cours de laquelle ETS1 a été réintroduit dans des cellules OCI-LY7 traités avec le shRNA de ETS1. Cette expérience a montré que la réintroduction de ETS1 restaurait l’expression d’ARN messager de PRDM1 à des niveaux comparables aux cellules contrôle (shGFP). Puisque des sites de liaison de ETS1 ont été trouvés dans le promoteur de PRDM1, une analyse de l’immunoprécipitation de chromatine (ChIP) a été conduite avec un anticorps dirigé...
contre la protéine ETS1 sur des cellules OCI-LY7 dont l’expression d’ETS1 ou du contrôle GFP ont été contrariées. Un enrichissement de la région promotrice de PRDM1 a été détecté dans le contrôle OCI-LY7 dans lequel le niveau d’expression d’ETS1 est élevé à cause du gain 11q24.3, alors qu’un enrichissement réduit du promoteur de PRDM1 a été observé dans les OCI-LY7 dont l’expression de ETS1 est réduite. Tous ces résultats montrent de façon concomitante que ETS1 régule directement et négativement la transcription de PRDM1. Nous n’avons pas observé le même effet sur l’expression de PRDM1 dans les autres lignées DLBCL sans gain du à des altérations génomiques du gène PRDM1 ou de son régulateur négatif BCL6 qui affectent toutes les lignées DLBCL analysées.


Pour conclure, le gain 11q24.3 détermine une expression élevée d’ETS1 et FLI1 qui pourrait contribuer au phénotype néoplasique en soutenant la croissance cellulaire et la déréglementation du programme transcriptionnel normal des cellules B du centre germinatif. Nos résultats suggèrent qu’ETS1 agirait principalement en bloquant les derniers stades de la différenciation des cellules B à travers la suppression directe de l’expression de PRDM1, FLI1 agissant comme un facteur de survie. De plus, ETS1 et FLI1 semblent réguler l’expression de gènes importants pour l’interaction des cellules B avec le microenvironnement du centre germinatif, renforçant ainsi leur rôle dans la genèse des lymphomes DLBCL.

A la suite de la publication de ces résultats montrant que le silencing de FLI1 était toxique pour toutes les lignées DLBCL, nous avons eu l’opportunité de collaborer avec le groupe de recherche dirigé par Jeffrey Toretsky (Georgetown University, Lombardi Comprehensive Cancer Center, Washington DC, USA). Le Dr Toretsky nous a fait parvenir le YK-4-279, une molécule inhibitrice d’EWS-FLI1, pour pouvoir évaluer son effet sur les lignées DLBCL. Les analyses préliminaires montrent un effet toxique de YK-4-279 sur tous les DLBCL testés.
Un deuxième objectif a finalement été poursuivi dans cette thèse. Parce que l'analyse génomique est importante pour le pronostic des patients DLBCL et le type de thérapie à utiliser et parce que l'implication clinique du gain du nombre de copies de MYC n'a pas été elucidé, une analyse du statut du gène MYC a été effectuée sur la cohorte des 166 patients DLBCL traités avec R-CHOP. Cette analyse n’a révélé aucune corrélation entre le gain du nombre de copies de MYC et le pire OS chez les patients DLBCL.
# Table of Contents

**Abbreviations** ............................................. I-III

**Preface** ..................................................... 3

**Introduction** ............................................... 5

1. **DIFFUSE LARGE B CELL LYMPHOMA** .......................... 6

2. **DEVELOPMENT OF MATURE B CELLS** .......................... 11
   2.1. Early B cell development ............................................ 12
       2.1.1. Generation of B cells in bone marrow ....................... 12
       2.1.2. VDJ recombination of immunoglobulin genes .................. 15
    2.2. Germinal center B cell ............................................. 17
       2.2.1. Germinal center microenvironment ......................... 17
       2.2.2. DNA modifications and lymphomagenesis .................... 21
          2.2.2.1. Somatic hypermutation (SHM) ......................... 22
          2.2.2.2. Class switch recombination (CSR) ...................... 23
       2.2.3. Role of chemokines in GC formation and maturation ....... 26
    2.3. Plasma cell differentiation and regulation .................... 26
       2.3.1. GC reactions ................................................. 26
          2.3.1.1. ETS1, v-ets avian erythroblastosis virus E26 oncogene homolog 1 ................................. 30
          2.3.1.2. FLI1, friend leukemia insertion 1 ....................... 33
       2.3.2. Plasma cell differentiation ................................... 36
    2.4. Remarks ..................................................... 38

3. **GENETICS OF DIFFUSE LARGE B CELL LYMPHOMA** ................. 40
   3.1. Germinal centre B cell-like diffuse large B cell lymphoma .......... 41
      3.1.1. BCL2 chromosomal translocation ......................... 411
      3.1.2. MYC deregulation ........................................... 422
      3.1.3. Chromatin remodelling ....................................... 43
      3.1.4. BCL6 deregulation ........................................... 466
      3.1.5. Other lesions .............................................. 466
    3.2. Activated B cell-like diffuse large B cell lymphoma .............. 48
      3.2.1. B cell differentiation ....................................... 488
      3.2.2. BCR and NF-KB signaling ................................... 499
      3.2.3. Cell cycle .................................................. 50
      3.2.4. Other lesions .............................................. 50
    3.3. Shared lesions ................................................ 50
      3.3.1. Immune surveillance ......................................... 50
      3.3.2. Aberrant somatic hypermutations (ashm) ................... 522
3.4. PMBCL .................................................................................................................. 533
3.5. Unclassifiable cases of B cell lymphoma with feature intermediate between DLBCL and BL .......................................................... 544
3.6. Remarks .............................................................................................................. 555

4. NEW AGENTS IN DIFFUSE LARGE B CELL LYMPHOMA PATIENTS  566
  4.1. Monoclonal antibodies (mAbs) ........................................................................ 577
  4.2. Immunomodulatory drugs ................................................................................ 599
  4.3. Cytotoxic agents ................................................................................................ 60
  4.4. Inhibitors of signaling pathways ...................................................................... 60
  4.5. PI3K/AKT/mTOR pathways inhibitors .............................................................. 611
  4.6. Kinase inhibitors ............................................................................................... 622
  4.7. Proteasome inhibitors ....................................................................................... 633
  4.8. Epigenetic inhibitors ......................................................................................... 644
  4.9. BCL2 inhibitors .................................................................................................. 655
  4.10. Immune checkpoints inhibitors ...................................................................... 666
  4.11. Remarks .......................................................................................................... 677

Purposes .................................................................................................................. 688

Materials and methods .......................................................................................... 722

Results ...................................................................................................................... 85

PRIMARY AIM
1. CHARACTERIZATION OF A NEW RECURRENT GAIN AT 11q24.3 GENOMIC LOCUS OF DLBCL PATIENTS CONTAINING TWO INTERESTING ETS FACTORS ................................................................. 86
  1.1. 11q24.3 gains a recurrent lesion in a subset of DLBCL patients ....................... 86
  1.2. ETS1 and FLI1 are the two targets of 11q24.3 gain ........................................ 87

2. FUNCTIONAL ANALYSIS OF ETS1 AND FLI1 IN A DLBCL CELL LINE CARRING THE GAIN ................................................................................................................................. 91
  2.1. OCI-LY7 is the best DLBCL cell line to study the role of ETS1 and FLI1 ...... 91
  2.2. ETS1 and FLI1 down-regulation using different methods ............................... 92
  2.3. ETS1 and FLI1 sustain OCI-LY7 cell viability and proliferation .................... 95
3. ETS1 AND FLI1 DOWN-REGULATION IN OTHER DLBCL CELL LINES ______98

4. ANALYSIS OF ETS1 AND FLI1 TARGETS IN DLBCL CELL LINES ______ 100
   4.1. ETS1 regulates genes involved in B cell differentiation in OCI-LY7 DLBCL cell line___ ________________________________100
   4.2. ETS1 and FLI1 transcription factors are involved in GC reactions ______104
   4.3. ETS1 directly regulates chemokine receptor CXCR5 ________________106

5. A NEW DRUG BLOCKING EWS-FLI1 ONCOGENIC PROTEIN HAS CYTOTOXIC EFFECTS AGAINST DLBCL CELL LINES ____________108

SECONDARY AIM

1. GAINS OF MYC LOCUS ARE NOT ASSOCIATED WITH POOR OUTCOME IN DLBCL PATIENTS _________________________________110

Discussion ____________________________________________112

DISCUSSION OF THE PRIMARY AIM OF THE THESIS___________ 113
DISCUSSION OF THE SECONDARY AIM OF THE THESIS_________ 123

Testoni M et al. BJH 2011 _________________________________ 134

Bibliography ____________________________________________138

List of publications ______________________________________154

Curriculum Vitae _________________________________________155
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC-DLBCL</td>
<td>activated B cell-like DLBCL</td>
</tr>
<tr>
<td>AHSCT</td>
<td>allogeneic hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>ASC</td>
<td>antibody secreting cells</td>
</tr>
<tr>
<td>ASHM</td>
<td>aberrant somatic hyper mutations</td>
</tr>
<tr>
<td>avBET</td>
<td>bromodomain and extra-terminal proteins</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt lymphoma</td>
</tr>
<tr>
<td>BLL</td>
<td>Burkitt-like lymphoma</td>
</tr>
<tr>
<td>BRD</td>
<td>bromodomain</td>
</tr>
<tr>
<td>Btk</td>
<td>bruton tyrosin kinase</td>
</tr>
<tr>
<td>C region</td>
<td>constant region (immunoglobulin protein)</td>
</tr>
<tr>
<td>CC</td>
<td>consensus cluster</td>
</tr>
<tr>
<td>CGP</td>
<td>chemical and genomic perturbations</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>cHL</td>
<td>classical Hodgkin lymphoma</td>
</tr>
<tr>
<td>CHOP</td>
<td>cyclophosphamide, doxorubicine, vincristine and prednisone</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid precursor</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>COO</td>
<td>cell of origin</td>
</tr>
<tr>
<td>CP</td>
<td>canonical pathway</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
<tr>
<td>CSR</td>
<td>class switch recombination</td>
</tr>
<tr>
<td>CTCL</td>
<td>cutaneous lymphomas</td>
</tr>
<tr>
<td>DLBCL</td>
<td>diffuse large B cell lymphoma</td>
</tr>
<tr>
<td>EBS</td>
<td>ETS binding site</td>
</tr>
<tr>
<td>ES</td>
<td>Ewing’s sarcoma</td>
</tr>
<tr>
<td>ETS1</td>
<td>v-ets avian erythoblastosis virus E26 oncogene homolog 1</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FFP</td>
<td>free from progression</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FL</td>
<td>follicular lymphoma</td>
</tr>
<tr>
<td>FLI1</td>
<td>friend leukemia insertion 1</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GCB-DLBCL</td>
<td>germinal center B cell-like DLBCL</td>
</tr>
<tr>
<td>GEP</td>
<td>gene expression profile</td>
</tr>
<tr>
<td>GSEA</td>
<td>gene set enrichment analysis</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venules</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HMT</td>
<td>histone methyltransferases</td>
</tr>
<tr>
<td>HR</td>
<td>host inflammatory response signature</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>ID-DLBCL</td>
<td>immunodeficiency DLBCL</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IPI</td>
<td>international prognostic index</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCL</td>
<td>mantle cell lymphoma</td>
</tr>
<tr>
<td>MCR</td>
<td>minimal common region</td>
</tr>
<tr>
<td>MHC I</td>
<td>major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MMr</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MZL</td>
<td>marginal zone lymphoma</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin Lymphoma</td>
</tr>
<tr>
<td>NLPHL</td>
<td>nodular lymphocytic predominant Hodgkin lymphoma</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>OxPhos</td>
<td>oxidative phosphorylation signature</td>
</tr>
<tr>
<td>PC</td>
<td>plasma cell</td>
</tr>
<tr>
<td>PFS</td>
<td>progression-free survival</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PIC</td>
<td>protein inhibitor cocktail</td>
</tr>
<tr>
<td>PMBCL</td>
<td>primary mediastinal B cell lymphoma</td>
</tr>
<tr>
<td>PRC2</td>
<td>polycomb repressive complex 2</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylycerine</td>
</tr>
<tr>
<td>PT-DLBCL</td>
<td>post-transplant DLBCL</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>R-CHOP</td>
<td>Rituximab plus CHOP</td>
</tr>
<tr>
<td>R-IPI</td>
<td>Rituximab-IPI</td>
</tr>
<tr>
<td>RS</td>
<td>Richter syndrome</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SHM</td>
<td>somatic hypermutation</td>
</tr>
<tr>
<td>SLL</td>
<td>small lymphocytic lymphoma</td>
</tr>
<tr>
<td>SSC</td>
<td>sclerosis or scleroderma</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TFH</td>
<td>follicular helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UNG</td>
<td>uracil DNA glycosilase</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>V region</td>
<td>Variable region (immunoglobulin protein)</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Non-Hodgkin’s lymphomas (NHLs) are the fifth most common cancer type in men and women. NHLs are heterogeneous lymphoproliferative malignancies deriving from T and B lymphocyte dysfunction.

B-cell non-Hodgking lymphomas (B-NHLs) are subdivided in several categories with different clinical behaviour and biology. Most of the categories derive from cells that are blocked within or have pass through the germinal center (GC). Thus, it is axiomatic that a component of the biology of a malignant cell is inherited from its normal cellular ancestors, and all NHLs are derived from B-lineage cells that have completed both immunoglobulin (Ig) heavy-chain and light-chain (IgH and IgL) recombination and can express functional Ig protein.

Diffuse large B cell lymphoma (DLBCL) is the most frequent B-NHL accounting for roughly 35-40% of all cases. DLBCL is an aggressive disease and treatments include anthracycline-based combination polichemotherapy (CHOP regimen), adoptive immunotherapy and bone marrow transplantation. Therefore, a dramatic increase in the cure rate for DLBCL patients came with the addition of rituximab (anti-CD20 monoclonal Ab) to the combination of CHOP (R-CHOP). However, about 50% of patients still die because of primary refractory disease or relapses after first-line therapy.

The modest therapeutic success is in part due to the remarkable heterogeneity of this disease, which can be appreciated at the morphologic, immunophenotypic, genetic and clinical levels. Advances in genomic research led to the characterization of at least three distinct biological subgroups of DLBCL, termed germinal center B cell-like (GCB) DLBCL, activated B cell-like (ABC) DLBCL, and primary mediastinal B cell lymphoma (PMBCL). GCB-DLBCLs seem to arise from highly proliferating B cell located within the GCs, in fact this DLBCL molecular subgroup harbors the typical expression pattern of GC B cells such as CD10 and BCL6 gene expression. ABC-DLBCLs may arise from post-GC B cells that are arrested during plasmacytic differentiation as suggest by the expression of NF-kB target genes caused by NF-kB constitutive activation. Finally, PMBCLs might arise from thymic B cells and is closely related to classical-Hodgkin lymphoma (cHL) in term of gene expression pattern and chromosomal lesions.

Arising from different B cells each subgroup is characterized by distinct oncogenic pathways and chromosomal aberrations and has significantly different overall survival (OS) (60% 5-year survival for GCB and 35% 5-year survival for ABC) following
chemotherapy. Thus, it is essential to better define all features of this disease to possibly identify therapeutic targets to increase cure rate of the patients. For this reason, the overall goal of this thesis is the identification and the functional characterization of a new genetic lesion recurrently found in DLBCL patients in order to add informations that better define DLBCL pathogenetic mechanisms.

This thesis is structure as followed: in the introduction I will describe all the mechanisms that characterize the development of normal B cells focusing on the network of GC reactions that allow an antigen-encountered B cell to differentiate into PC. Then, I will present the known genetic aberrations characterizing each subgroups for their implications in the prognosis and therapy of DLBCL patients. Finally I will describe the several drugs currently used to treat DLBCL cases. After the introduction I will start with my own work in order to show my practical work that I carry on during my PhD experience. My work include two aims, not related to each other, corresponding to the two published articles in which I contributed as first author. The main aim was the functional characterization of the 11q24.3 gain in DLBCL, of which the title of this thesis. The secondary aim was a short analysis of the association between MYC gene status and the overall survival of DLBCL patients.

Importantly, in this thesis I tried to underline also the biological significance of my work that in part has already been appreciated with the starting of collaboration with Toretsky group in Washington DC, USA.
Introduction
1. *Diffuse Large B Cell lymphoma*
Diffuse Large B cell Lymphoma (DLBCL) is a neoplasm of proliferating large blastic cells, which harbor a B cell phenotype. DLBCL is the most common Non-Hodgkin Lymphoma (NHL), accounting for 35%-40% of all cases [1-3]. DLBCL represents a very heterogeneous group of tumors. DLBCL can arise in a nodal (lymphoid) or extranodal site, including unusual sites, such as testis, bone, and lung, and tends to have an aggressive biological nature, but is highly responsive to combination chemotherapy. DLBCL can also occur in the setting of immunodeficiency, primary or acquired in recipients of solid organ transplants or in individuals with Human Immunodeficiency Virus (HIV) infection [4, 5]. DLBCL usually arises de novo but can represent transformation of an indolent lymphoma, such as follicular lymphoma (FL) [6, 7], chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) in the so-called Richter’s transformation [8], and marginal zone lymphoma (MZL) or nodular lymphocytic predominant Hodgkin lymphoma (NLPHL) [9]. Anthracycline-based combination chemotherapy combined with anti-CD20 monoclonal antibody rituximab represents one of the successes in the treatment of lymphoma patients. Nevertheless, 50% of the DLBCL patients cannot be cured yet because of primary refractory disease or relapses after first-line therapy [2, 10, 11].

Before the introduction of rituximab, the International Prognostic Index (IPI) has been the most important tool used to predict the response to treatment and prognosis of patients with DLBCL [12]. This indicator identified five adverse risk factors in patients with aggressive NHL [12]. The risk factors — age older than 60 years, poor performance status, elevated LDH, advanced Ann Arbor stage, and presence of more than one extranodal site — identify four risk groups with 5-year overall survival (OS) ranging from 73% in the most favorable group to 26% in the last. However, The addition of rituximab to standard CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisolone) has led to a marked improvement in survival and has restricted the outcome differences between the IPI risk groups questioning the significance of previously recognized prognostic markers [3, 13]. The revised rituximab-IPI (R-IPI), assessed with a retrospective analysis of DLBCL patients treated with R-CHOP, identified 3 different risk groups with a more accurate prediction of outcome [353]. Therefore, a variability in the outcome of DLBCL patients remains to be observed and this variability reflects molecular heterogeneity in the tumors. In fact, these models based on risk factors do not provide specific insights regarding tumor cell biology, novel therapeutic targets, or more effective treatment strategies.

Acknowledging the heterogeneity of DLBCL, the emphasis in recent years has focused on pathways of transformation and molecular alterations as a way to further subclassify...
these tumors into biological relevant subgroups for which specific targeted therapies could be used. Gene expression profiling (GEP) has identified different DLBCL subtypes, bearing prognostic significance [14]. At least three main subtypes of DLBCL were initially identified based on the cell of origin (COO) of the groups, all with underlying clear clinical, biologic and genetic peculiarities [11, 14-16]: germinal centre B cell-like (GCB) DLBCL, expressing genes that are hallmarks of normal germinal center B cells; activated B cell-like (ABC) DLBCL, lacking expression of germinal center B cell-restricted genes and possibly arising from post-germinal center B cells that are arrested during plasmacytic differentiation; primary mediastinal B cell lymphoma (PMLBCL), expressing several genes that characteristically expressed also in Hodgkin Reed-Sternberg cells, and possibly derived from thymic B cells. The latter represents less than 10% of all DLBCL and is now considered a separate entity in the WHO classification [1].

![Figure 1: Survival rates for patients with a molecular diagnosis of the GCB or ABC subtype after R-CHOP therapy [11].](image)

This analysis has been used to highlight similarities between subsets of tumors and normal B cell counterpart and to identify features associated with unfavorable responses to combination chemotherapy. In fact, the use of GEP analysis allowed a better understanding of the molecular mechanisms underlying the development of this disease,
Introduction – Diffuse large B cell lymphoma: State of the art

but also revealed a number of features associated with an unfavorable clinical outcome [11, 14, 17]. ABC-DLBCL have a worse outcome than GCB-DLBCL (Fig.1.), and, as we will see below clear genetic and biologic differences [11, 14, 15, 18].

In addition to the molecular characterization of DLBCL, also biologic attributes have been identified using GEP analysis that influence the OS of patients [19]. A gene expression-based survival predictor model consisting of three gene expression signatures termed “germinal center B cell”, “stromal-1”, “stromal-2” can divide DLBCL patients into prognostically different groups. These signatures are integrated with a statistical model in which the risk for each patient is estimated by a survival predictor score. The “germinal center B cell” signature mirrors the distinction between GCB and ABC, while the stromal-1 and “stromal-2 signatures reflect the composition of the tumor microenvironment. The favorable signature “stromal-1” reflects extracellular matrix deposition and infiltration of the tumor with macrophages, conversely, the unfavorable “stromal-2” signature reflects tumor with a high density of blood vessels [11, 19].

In a study by Monti et al. three profiles characterize DLBCLs by the expression of genes involved in oxidative phosphorylation (OxPhos), which harbours the signature of genes involved in mitochondrial metabolism; B cell receptor signaling (BCR), which has more abundant expression of cell cycle regulatory genes, DNA repaired genes, components of B cell receptor signaling cascade and B cell specific transcription factors; and host inflammatory response (HR), enriched in genes of T cell-mediated immune response, classical component pathway and inflammatory response [20].

Comparison of the consensus cluster (CC) [20] and cell of origin (COO) [14] molecular signatures indicates that the two classification schemes capture different aspects of DLBCL biology [20].

The CC classification has no prognostic relevance but might have therapeutic implication. For example, DLBCLs of OxPhos signature may be particularly sensitive to proteosome blockade or BCL2 family inhibition, because their increased expression of proteosomal subunits and molecules regulating mitochondrial membrane potential and apoptosis. In contrast, tumors in the HR group show increased expression of macrophage/dendritic cell markers, T/natural killer cell receptor and activation pathway components, as well as complement cascade members, and inflammatory mediators, suggesting an increased inflammatory response. Clinically this tumor is less responsive to CHOP-based treatment and this means that it will be important to identify HR tumors with pre-existing abundant T- and dendritic cell infiltrates and further characterize their associated underlying immune response [21].
Introduction – Diffuse large B cell lymphoma: State of the art

Because it is currently impractical to perform microarray analysis on every patient with DLBCL, immunohistochemical profiles analysis have been proposed to divide DLBCL patients into prognostic groups based on the cell of origin of the tumor (Table 1) and as surrogate of GEP [13, 22-29].

A few studies have used the immunohistochemical expression of CD10, BCL-6, or MUM1 to classify cases of DLBCL into GCB and non-GCB groups, but without a direct comparison with microarray, leading to conflicting results [30, 31]. The algorithms of Choi [32] and Hans [33] appear the best ones in predicting cell of origin as defined by GEP and in predicting the outcome [25].

In the study by Hans et al. [33] sections were stained with antibodies against CD10, BCL-6, MUM1, FOXP1, cyclin D2, and BCL2 [33], and expression of BCL6 (P <.001) or CD10 (P =.019) were associated with better OS, whereas expression of MUM1 (P =.009) or cyclin D2 (P <.001) were associated with worse OS in non-GCB DLBCL patients.

The study by Choi et al. [32] added two GC-specific markers, GCET1 and MTA3, and one ABC marker, FOXP1 [32].

<table>
<thead>
<tr>
<th>Algorithms</th>
<th>CGB-DLBCL</th>
<th>ABC-DLBCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choi</td>
<td>GCET1 &gt;80% CD10 &gt;10% BCL6 &gt;30%</td>
<td>MUM &gt;80% FOXP1 &gt;80%</td>
</tr>
<tr>
<td>Hans</td>
<td>CD10 &gt;30% BCL6 &gt;30%</td>
<td>MUM1 &gt;30%</td>
</tr>
<tr>
<td>Nyman</td>
<td>CD10 &gt;30%</td>
<td>MUM1 &gt;30% FOXP1 &gt;80%</td>
</tr>
<tr>
<td>Muris</td>
<td>CD10 &gt;30%</td>
<td>MUM1 &gt;30% BCL2 &gt;50%</td>
</tr>
<tr>
<td>Tally</td>
<td>GCET1 &gt;80% CD10 &gt;10% LMO2 &gt;30%</td>
<td>MUM &gt;30% FOXP1 &gt;80%</td>
</tr>
<tr>
<td>Natkunam</td>
<td>LMO2 &gt;30%</td>
<td>FOXP1 &gt;60%</td>
</tr>
<tr>
<td>Visco</td>
<td>CD10 &gt;30% BCL6 &gt;30%</td>
<td>FOXP1 &gt;60%</td>
</tr>
</tbody>
</table>

*Table 1*: Markers used in different algorithms to predict DLBCL cell of origin (COO).
2. Development of mature B cells
2.1. EARLY B CELL DEVELOPMENT

2.1.1. GENERATION OF B CELLS IN BONE MARROW

In mammals B cells development occurs mainly in the specialized environments of the central lymphoid organs, the bone marrow for the adults and the liver in the fetus. Lymphocytes develop from a common lymphoid precursor (CLP), who derives from pluripotent hematopoietic stem cells (HSC) that reside in the bone marrow. The CLP could give rise to T cells, B cells and NK cells but not to other cell types [34]. Development involves the successive acquisition of properties that are essential for the function of the mature cells along with the loss of properties that are more characteristic of the immature cell.

B cell development is dependent on the non-lymphoid stromal cells of the bone marrow and this contribution is two-fold. First, stromal cells form specific adhesive contacts with the developing B lineage cells by interactions between cell adhesion molecules and their ligands. Second, they provide soluble factors that control lymphocyte differentiation and proliferation, like stem cell factor (SCF), a cytokine that interacts with the cell-surface receptor tyrosine kinase kit on B cell precursors and induces the proliferation. The chemokine CXCL12 (stromal cell-derived factor 1, SDF-1) is also essential for the early stages of B cell development. It is produced constitutively by the stromal cells, and one of its roles may be to retain developing B cell precursors in the marrow microenvironment [35]. As B lineage cells mature, they migrate within the bone marrow, remaining in contact with the stromal cells (Fig.3).

The stages in primary B cell development are defined by sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes (Fig.4). This phenomenon, called VDJ rearrangement, implies a highly regulated series of genetic events resulting in the membranous expression of a functional B cell receptor (BCR) [36]. The BCR is composed of two identical heavy-chain and two identical light-chain Ig polypeptides that are covalently linked by disulphide bridges forming the Ig surface molecule. Each heavy- and light- chain have two regions, the constant region (C region) and the variable region (V region) that are modified during B cell maturation process. Other components of the BCR are the CD79A (also known as Igα) and CD79B (Igβ) molecules, which contain cytoplasmic immune receptor tyrosine-based activation motifs.

The earliest B lineage cells are known as pro-B cells, because they are progenitor cells committed to the B cell lineages. They are derived from HSC and are identified by the appearance of cell-surface proteins (e.g. CD19 and CD79a) characteristic of early B lineage cells and by their ability to start VDJ rearrangement.
Productive VDJ joining leads to the expression of an intact µ heavy chain, which is the hallmark of the next main stage of development, the pre-B cell stage.

As B cells develop from pro-B cells to mature B cells, they express proteins other than Ig that are characteristic of each stage. One of the first identifiable proteins expressed on the surface of B cell is CD42R. This is a B cell-specific form of the CD45 protein; T cells, monocytes, and neutrophils express other variants of this protein. D45R is a tyrosine phosphatase required for receptor signaling in B cells. Another protein is CD19, which also participates in B cell receptor signaling, and CD43, which functions both as an adhesion molecule that may guide cell-cell interactions, for example those of B cell precursors with stromal cells, and also as a signaling molecule, although not as part of the BCR complex. Kit, the receptor for bone marrow stromal cells, is highly expressed on stem cells and in the late pro-B cell stage and Kit signaling promotes the proliferation of pro- and pre-B cells.

Figure 2: Lymphocytes derive from a common lymphoid precursor. In the bone marrow the pluripotent stem cell give rise to cells with progressively more limited potential. The first branch leads to cells of myeloid and erythroid potential on the one hand and lymphoid potential on the other hand. The lymphocyte progenitor can give rise to NK cells, T cells or B cells through successive stages of differentiation in either the bone marrow or thymus [34].
Figure 3: The early stages of B cell development are dependent on bone marrow stromal cells. The upper panels show the interaction between precursors B cells and stromal cells that are required for the development to the immature B cell stage. The lower panel shows high-magnification electron micrograph of a similar cell culture in which two lymphoid cells are seen adhering to a flattened stromal cell [37].

Figure 4: The development of a B-lineage cell proceeds through several stages marked by the rearrangement and expression of the immunoglobulin genes. The heavy-chain locus rearrange first. Rearrangement of light-chain locus starts later when the cells are again small. Upon successfully assembling a light-chain gene, a cell becomes an immature B cell that expresses a complete IgM molecule at the cell surface. Mature B cells produced a δ heavy chain as well as a µ heavy chain, by a mechanism of alternative mRNA splicing, and are marked by the additional appearance of IgD on the surface [37].
2.1.2. VDJ RECOMBINATION OF IMMUNOGLOBULIN GENES

The V region, or V domain, of an Ig heavy- or light-chain is encoded by more than one gene segment. For the light-chain there are V gene segments (denoted V_{L}) that encode most of the V domain, and joining or J gene segments (J_{L}) that encode the remainder of the V domain. The joining of V and J gene segments create a continuous exon that encode the whole of the light-chain V region. The heavy-chain V region is encoded in three gene segments. In addition to the V and J gene segments (V_{H} and J_{H}), there is a third type of gene called diversity or D_{H} gene segment, which lies between the V_{H} and J_{H} genes.

There are three sets of Ig chains, the heavy-chain and two equivalent types of light-chain, the κ and λ chains, that are on different chromosomes (14, 2 and 22 respectively) and each is organized slightly differently with a diverse number of V, J and, for the heavy-chains, D gene segments (Fig. 5 upper panel.).

The recombination process is imprecise, however, with the random addition of nucleotides at the joins between gene segments. This means that it is a matter of chance to generate an out-of-frame sequence downstream from the join. Thus, B cell development has evolved to preserve and multiply those B cells that have made productive joins and eliminate cells that have not.

Assembly of the gene segments to produce a complete receptor requires three separate recombination events, which occur at different stages of B cell development. These are, in the order that they occur: the joining of D_{H} to J_{H} and V_{H} to DJ_{H} to produce the functional heavy-chain gene, and the joining of V_{L} to J_{L} to produce the functional light-chain gene (Fig. 5 lower panel).

A large number of developing B cells are lost because they fail to make a productive rearrangement at one of these stages (Fig. 5 lower panel.).

The complex of enzymes that act to carry out somatic V(D)J recombination is termed the V(D)J recombinase. The products of the two genes RAG-1 and RAG-2 (recombination-activating genes) comprise the lymphoid-specific components of the recombinase and are expressed only while they are engaged in assembling their antigen receptors. The remaining enzymes are ubiquitously expressed and they include the DNA ligase IV, the DNA-dependent protein kinase (DNA-PK), Ku, which is a heterodimer (ku70:ku80) tightly associated with DNA-PK, and Artemis, a protein involved in the opening of covalently closed DNA ‘hairpin’ structures. There is also a DNA repair enzyme in the complex that modifies the opened hairpin by removing nucleotides by exonuclease activity and by randomly adding nucleotides, and it is called TdT, terminal deoxynucleotidyl transferase.
Figure 5: Upper panel. The genetic locus for the \( \lambda \) light chain has about 30 functional \( V\lambda \) gene segments and four pairs of functional \( J\lambda \) gene segments and \( C\lambda \) genes. The \( \kappa \) locus is organized in a similar way, with about 40 functional \( V\kappa \) locus and a cluster of five \( J\kappa \) gene segments and a single \( C\kappa \) gene. The heavy-chain locus has about 40 functional \( VH \) gene segments and a cluster of around 25 \( DH \) segments lying between these \( VH \) and six \( JH \) gene segments. The heavy-chain also contain a cluster of \( CH \) gene segments, each of which corresponds to a different isotype. Lower panel, the developmental program rearranges the heavy-chain locus first and then the light-chain loci. Cells are allowed to progress the next step when a productive rearrangement has been achieved, so that fewer cells are lost during B cell development [37].

Once a light-chain gene has been rearranged successfully, light-chains are synthesized and combine with the heavy-chain to form intact IgM, and immature B cells can now interact with antigens in its environment. Immature B cell that are strongly stimulated by self-antigen at this stage either die or are inactivated in a process of negative selection, thus removing many self-reactive B cells from the repertoire. At the end, the surviving immature B cells emerge into the periphery and become a mature B cell to expressing IgD as well IgM on its surface. In contrast to immature B cell, mature B cell expresses a
Introduction – Development of mature B Cells

functional BCR. This cell, also called naïve B cell, can now be activated by encounter with its specific foreign antigen and differentiate into either plasma cell (PC) or memory B cell in a secondary lymphoid organ.

2.2. GERMINAL CENTER B CELL

2.2.1. GERMINAL CENTER MICROENVIRONMENT

To better understand the mechanisms of lymphomagenesis it is important to study the normal behavior of a B cell passing through the germinal center (GC). The first step of the humoral response is the exposure to a microbe or an antigen that leads to the activation of naïve B lymphocytes. This activation can happen in two ways: in a T cell independent manner or in a T cell dependent manner.

In the T cell dependent maturation process the CD4+ helper T lymphocytes stimulates B cells leading to a humoral immune response with antibodies of high affinity against protein antigens. In contrast, antibodies response to non-protein antigens does not need participation of a helper T cell generating antibodies with a lower affinity for the antigen. T cell dependent antigen responses can leads to two different B cell maturation processes. In fact, naïve B cells can be induced to proliferate and differentiate in either follicular or extrafollicular antibody-secreting cells. T cell dependent extrafollicular responses provide early antibody, which can be critical for halting the spread of viral infection [38]. In this condition, memory B cells are not produced. By contrast with the establishment of a primary follicle and, successively, a GC there is also a memory B cells production and affinity maturation of the BCR [38].

GC is formed from a small number of IgM+ IgD+ B cells in the follicles of peripheral lymphoid tissues (spleen, lymph node, Peyer’s patches and tonsils). Under experimental conditions, GCs initially form approximately 6 days after a primary immunization, when foci of rapidly proliferating B cells begin to appear within the B cell follicles of lymph nodes and spleen.

Naïve B cells that have taken up antigen through their BCRs, internalize it and after digestion expose it through the MHC class II (major histocompatibility complex class II) molecules. Thus, naïve B cells migrate into lymphoid tissue through high endothelial venules (HEV) and at first enter the T cell zones (Fig.6). Here, antigen-binding B cells are selectively trapped to maximize the chance of encountering a CD4+ helper T cell that can activate them. This interaction is managed by MHC class II molecules on the surface
of B cells associated with the antigen that is recognized by T cell receptor (TCR) on T cells surface.

**Figure 6:** GC development. Upon entry into the spleen naïve B cells, that have encountered the antigen, move to the T cell zone through the high endothelial venules (HEV) to be activated. Then, B cells move to B cell zone and start to proliferate establishing a primary follicle of clonal expansion and successively the GC. GCs are sites of rapid proliferation and differentiation follicles in which GCs have formed are known as secondary follicles. Here, B cells form two zones: dark zone and light zone in which B cells continue to migrate in a movement called “cyclic re-entry”. Within the GC, BCR increases affinity for the antigen and allows selection in the light zone together with CD40-CD40L signal. At the end of this process B cells differentiate into either antibody-secreting plasma cells (PC) or memory B cells. PCs leave the lymph node and migrate to the bone marrow. Memory B cells continue to recirculate through the B cell zone of secondary lymphoid tissue. FDC, follicular dendritic cell; TFH, follicular helper T cell; MΦ, macrophages.

Helper T cell that recognizes MHC:peptide complex on B cell also binds to B7 molecule with its CD28 ligand and gets stimulated to proliferate. BCR signalling is also enhanced by the presence of another important surface molecule CD40, which is a member of the tumor-necrosis factor (TNF)- receptor family that is expressed by all B cells. CD40 receptor
binds to its ligand CD154 (or CD40L) expressed by CD4+ helper T cells. This engagement results in the nuclear translocation of NF-κB that stimulates proliferation. Mice with no functional CD40 or CD40L do not develop GCs, and mechanistic disturbance of this interaction leads to a dissolution of formed GCs [39].

Once activated, B cells move through the T cell zone into the B cell zone where they start to proliferate forming a primary follicle of clonal expansion. Here, at the border between T cell and B cell zones, B cells massively continue to proliferate for several days constituting the GC.

In addition, after interaction between B and T cells, T cells are stimulated to produce cytokines like IL-4 that synergize with CD40L in driving the clonal expansion that precedes antibody production. Thus, the combination of BCR and CD40 ligation, along with IL-4 and together with others signals derived from T cell contact, lead to B cell proliferation. B cells that form GC are called centroblast. In this GC phase, centroblasts start to express BCL6, an important GC factor, and continue to proliferate, forming the characteristic phenotype of the GC with two visible zones: the dark and the light zones [40] (Fig. 6).

Studies in rat spleens indicate that, on average, GCs contain only three B cell clones and that each one grows to produce about 4000 cells within 96 hours. In the dark zone B cells become B blasts and stop the expression of CD40 molecule. This occurs because dark zone is largely devoid of T cells that express CD154 (CD40L molecule), and then is not necessary for B cells to expressed CD40 surface molecule, and the interaction between CD40 and CD154 does not happen [41]. In this way, NF-κB is not expressed or expressed at very low levels and this is in accordance with the fact that NF-κB is a repressor of BCL6 that is one of the master regulators of the early phase of GC.

Dark zone is so called because the proliferating B cells are densely packed, while the light zone is more supplied with follicular dendritic cells (FDC), follicular helper T cell (TFH) and macrophages but less densely packed with B cells (Fig. 6).

The high proliferation rate of centroblasts is required for the generation of large number of modified antibodies, and to allowed the selection of few B cells that display antibodies with improved antigen-binding specificity. Importantly, in the dark zone of centroblasts somatic hypermutation (SHM) occurs, which alters the V region of Ig genes leading to a high number of “new” B cells.

Gene expression profile analysis has shown that the differentiation of an antigen-activated B cell into a centroblast is accompanied by a dramatic up-regulation of genes associated with cell proliferation and the down-regulation of genes encoding negative regulators of clonal expansion, such as p21 [42]. In this context, DNA damages frequently occur, but in high proliferating B cells the sensing of and response to DNA damage are
specifically suppressed [43]. Furthermore, to allow that the vigorous clonal expansion does not result in a loss in the replicative potential, the enzyme telomerase is expressed [44]. Moreover, centroblasts express low levels of anti-apoptotic proteins such as BCL2 and NF-kB, but they express several pro-apoptotic molecules [41], which allow the rapid execution of cell death by default or in response to exogenous signals. Compared to centroblast, centrocytes are very heterogeneous and must undergo selection, further DNA modification and initiation of differentiation into PC or memory B cell. In the light zone selection of B cells expressing mutated BCR is essential to generate antibodies with high-potential affinity. Current evidence favours a two-stage process for centrocytes selection. First, the cells engage antigen through their BCR. In this phase the antigen is exposed by FDC and the selection is governed by direct signals derived from BCR crosslinking more strong in proportion to the affinity of the BCR [45-47]. This selection phase is thus based on affinity-like parameter that change with mutations. The second phase seems to involve the binding between CD40 expressed on centrocytes and CD40L expressed by TFH. Thus, in the light zone CD40 molecule is re-expressed on centrocytes leading to signals of "delivery of survival". B cells with a too low affinity for the antigen die because are not rescue by CD40 signal. Another important signal derives from IL-21 produced by TFH that induces PC differentiation from ongoing GC reactions, and promotes and maintains the TFH lineage [48, 49]. These signals provide the cell fate of mature B cells with the activation of the NF-kB factor that inhibits BCL6 and GC important genes and up-regulates differentiation pathways. Class switch recombination (CSR) is also induced leading to DNA modification with which B cells change Ig class expression from IgM and IgD to other classes that have distinct effectors function (IgG, IgA and IgE). Apart from CD40 signaling, cytokines too, expressing by TFH, play an important role in regulating the pattern of heavy-chain isotype switch. For example, IL-4 induces switch to IgE.

Dark zone and light zone are two GC compartments containing B cells that are usually seen as two different populations with a common precursor. But now different studies support the idea that centroblasts and centrocytes are the same B cells which differed for their status [50-53]. Gene expression profile analysis reveals that, rather than differentiation pathways, centroblasts and centrocytes differ for proliferation pathways more active in the dark zone, and for signalling pathways more active in the light zone where B cells encounter FDC and THF. Thus, dark and light zones seem to be different GC compartments specialized in proliferation and selection of B cells, idea supported also by the finding that centroblast and centrocytes have the same cell size and morphology, as determined by flow-cytometric forward-and side-scatter measurement [50, 51]. Based on
Introduction – Development of mature B Cells

this idea, it was described a nonrandom migration in GC forming circle between centroblast and centrocytes with a strong and net flux from dark to light zone, with only a few cells returning from light to dark zone, a model termed “cyclic re-entry” (Fig.6) [54]. This is been demonstrated using a long-term multiphoton live-imaging studies [51] supporting the idea of cycles of selection in the GC on the basis of competition for limited available signals in the light zone capable of promoting their survival and returning into the dark zone. The time course of this process is of 4-6 hours [51, 55]. The decision of a B cell to return into the dark zone to undergo further proliferation and mutation is controlled by T helper cells on the basis of the amount of antigen captured and presented by the GC B cell [51]. Evidence suggests that the differentiation of a GC B cell into PC is driven by acquisition of a high-affinity BCR [56]. Various culture conditions have been described that will induce PC differentiation from centrocytes in vitro, but the way in which centrocytes are induced to differentiate into PCs in vivo requires further study. In general, long-live PCs differentiation is linked to the number of rounds of GC B cell division. This association may in part be due to increased levels of SHM with continued centroblast divisions, and the tendency of higher-affinity GC B cells to be selected for PC differentiation. TFH-cell-derived IL4, IL21 and CD40L also impact the quality of PC responses through direct stimulation of GC B cells. The fate of memory B cells differentiation is still unclear but seem to be stochastic, as throughout GC formation, GC B cells are constantly selected to enter the memory pool [57].

2.2.2. DNA MODIFICATIONS AND LYMPHOMAGENESIS

GCs are sites of oligoclonal B cell growth. DNA modifications that affect Ig genes of B cells during GC phase include SHM, which alters the V region of Ig genes during the proliferation phase of centroblasts, and CSR that occurs in the light zone of centrocytes. All these mechanisms require DNA strand breaks process and the activity of activation-induced cytidine deaminase (AID), which is an enzyme that catalyses the deamination of cytidines directly in the VDJ and switch regions of the Ig genes, that is the starting point for DNA strand breaks [58, 59]. However, AID is not always specific for the Ig locus, and can cause mutations in oncogenes or double strand breaks leading to a genomic instability. These events importantly contribute to lymphomagenesis and I will describe them more in detail.
2.2.2.1. Somatic hypermutation (SHM)

The GC, dark zone in particular, is the main structure where SHM occur. The physiological role of SHM is to introduce point mutations in the V region of rearranged Ig genes. SHM leads to affinity maturation, which results in the preferential outgrowth of B cells expressing an Ig with high affinity for its cognate antigen [60]. The mutations introduced by SHM are predominantly point mutations, although insertions and deletions are occasionally observed. The base changes are distributed throughout the V region, but not completely randomly; there are ‘hotspots’ of mutations that indicate a preference for characteristic short motifs of four or five nucleotides, and, perhaps, for secondary structural features as well. Some of the mutant Ig molecules bind antigen better than the original BCR, and the B cell clones that express them are preferentially selected to mature into antibody-secreting cells.

The process of SHM is closely linked to transcription [60]. The mutation rate of an Ig gene is proportional to the transcription rate of the locus, and mutations are confined to a 1-2 kilobase (kb) region downstream of the transcription start site [60].

As mentioned before, AID is required for this process. This enzyme acts directly on DNA, converting cytidine (C) to uridine (U) in Ig V regions. After AID initiates SHM by the deamination of C nucleotides, the resulting U-G mismatch may lead to mutations in several different ways. If the mismatch is not repaired before the onset of DNA replication, DNA polymerases will insert an A nucleotide opposite to U, thus creating a C→T and G→A transition (interchanges of two ring purines, G→A, or one-ring pyrimidines, C→T). Conversely, if the U is removed by uracil DNA glycosilase (UNG), an abasic site is created, and its replication may give rise to either transitions (interchanges of two-ring purines, A-G, or one-ring pyrimidines, C-T) or transversions (interchanges of purine for pyrimidine bases, which therefore involve exchange of one-ring with two-ring structure). In addition, U-G mismatch recruits the mismatch repair (MMR) machinery, which creates mutations at A-T near the initiating U-G lesion.

The rate of mutation in the Ig V regions during SHM in the GC is estimated to be as high as 103 mutations per base pair per generation [61, 62].

As above mentioned, SHM is not strictly limited to Ig genes, but, in normal B cells, it targets also other genes expressed in the GC, including, for example, BCL6 and CD95/FAS [63, 64]. In some lymphomas deriving from GC B cells, not only DLBCL, SHM may target several proto-oncogenes, thus contributing to neoplastic transformation. Malfunctioning of SHM in the context of lymphoma is termed aberrant SHM and the proto-oncogenes most frequently affected include BCL6, PIM1, MYC, RHOH (Ras homologue gene-familily member H), and PAX5 [65-67]. Aberrant SHM can contribute to
lymphomagenesis by mutating both regulatory and coding sequences in proto-oncogenes \[65-67\]. For example, mutations in MYC frequently target important functional domains and regulatory sequences, and mutations in the BCL6 regulatory region have been shown to deregulate BCL6 expression \[68-70\]. Also, SHM may promote chromosomal translocations of the targeted proto-oncogenes in the regions where SHM-derived mutations are located.

2.2.2.2. Class switch recombination (CSR)

During CSR of the membrane-expressed BCR, which is primarily encoded during development and maturation, the C regions are replaced. In particular, this modification replaces the expressed \( \mu \) constant region \((C_\mu)\) of the heavy-chain locus with a downstream isotype \(C_7, C_\alpha,\) or \(C_e\). The process alters the effectors functions of an antibody. CSR is an irreversible somatic recombination mechanism by which B cells can switch their Ig class expression from IgM and IgD to IgG, IgA and IgE. CSR occurs via DNA recombination involving non-homologous end-joining processes between specific repetitive regions of several hundred base pairs (known as switch regions) that precede the Ig constant region genes. CSR, as well as SHM, is initiated by the activity of AID selectively expressed in GC B cells \[71\] and it can occur in both T cell dependent and T cell independent manner. It is the combination of specific cytokines and co-stimulatory signals that determine the nature of resulting Ig class. Different cytokines that regulate heavy-chain class switching are made by different subsets of helper T cells that are generated in response to distinct types of microbes. TGF-\( \beta \) that is produced by many cell types, in association with T cell derived IL-5 stimulates production of IgA in mucosal lymphoid tissue, resulting in production of local immunity. IL-4 alone is necessary for IgE, while when in association with IL-6, IL2 and INF-\( \gamma \) induces IgG switch.

For CSR, CD40-CD154 interaction is critical, and IRF4, a downstream target of NF-kB, regulates AID expression through intermediate molecules rather than through a direct mechanism \[71\].

A hallmark of mature B cell lymphomas is the presence of reciprocal chromosomal translocations that juxtapose loci encoding the Ig genes to a proto-oncogene. In the process of CSR, DNA double-strand break intermediates generated in Ig genes by this reaction are potent substrates for translocations by recombining with double-strand breaks on a nonhomologous chromosome. The formal proof that AID is required for GC-derived lymphomagenesis is based on the observation that AID deficiency prevents the formation of BCL6-dependent GC-derived B cell NHL in lymphoma prone animal models \[72\]. Regarding DLBCL, some of the most commonly translocated genes are: BCL2, BCL6
and c-MYC, whose coding domain undergoes under the control of Ig promoter, leading to deregulation of their expression.

Translocations t(14;18)(q32;q21) involving the Ig heavy-chain locus and the BCL2 proto-oncogene are characteristic of follicular lymphoma (FL). As centroblasts do not normally express BCL2, the ectopic expression of BCL2 in these cells promotes lymphomagenesis by overriding the characteristic pro-apoptotic program of the centroblast. In contrast to BCL2, BCL6 is normally expressed in GC B cells, but its expression has to be switched off for the post-GC differentiation of B cells to take place. Chromosomal translocations involving the BCL6 gene locus (3q27) is commonly associated with 30-40% of DLBCL and less frequently with FL and it causes the deregulation of BCL6 expression by preventing its silencing at the conclusion of the GC response. The BCL6 translocation might result in a block in PC differentiation maintaining GC conditions with high proliferation rate, DNA-damage tolerance with the possible consequence that the cells may be subjected to further genetic alterations, eventually contributing to lymphomagenesis.

Translocations of MYC into the Ig heavy- or light-chain loci are associated with 100% of Burkitt lymphoma (BL) cases and up to 10% of DLBCL cases. Normally, GC B cells do not express MYC and this means that expression of MYC in BL and DLBCL is ectopic. MYC deregulation has been associated with abnormal cell growth, as well as genomic instability. Thus, it is conceivable that the ectopic expression of MYC in GC B cells contributes to lymphomagenesis.

I will come back to the chromosomal translocations occurring in DLBCL in the next chapter.

2.2.3. ROLE OF CHEMOKINES IN GC FORMATION AND MATURATION

Lymphocytes can migrate toward and between tissue different compartments. This movement, or homing, is regulated by chemokines and the stromal cells network in the lymphoid microenvironment. Lymphocytes subsets specifically program their expression of chemokines receptors, selectively allowing the movement to specific functional compartments of the immune system. Thus, migration is essential for tissue morphogenesis and leukocyte trafficking, as during GC formation and development.

B cells passage from blood into lymph nodes is a multistep process involving chemokines and receptors. First, lymphocytes are recruited from HEVs via adhesive interaction between the selectins on B cells and their receptors on HEVs. Then, B cells trigger thanks to chemokines receptors on their surface. Chemokine receptors are Gi protein–coupled
receptors that induce integrin-dependent firm adhesion. Once firm adhesion has been achieved, the B cells proceed through an additional series of steps that lead to migration into the lymph node.

Circulating B and T cells express multiple chemokine receptors including CXCR7, the receptor for CCL19 (ELC) and CCL21 (SLC), CXCR4, the receptor for CXCL12 (SDF1), and, especially for B cells, CXCR5, the receptor for CXCL13 (BLC) [72]. CCL21 and CCL19 are expressed at high levels by HEVs[73]. T cells lacking CXCR7 are markedly suppressed in their ability to enter wild-type lymph nodes [74, 75], and similar effects are seen also for B cells [76], but CXCR7 role in B cells is not well understood yet.

After the contact with T cells, newly activated B cells migrate to the B cell zone distal to T cell zone and start the clonal expansion forming GC (Fig.6). The physiological division in the GC between dark zone and light zone, selection and affinity maturation of B cells requires a movement between the two zones and these discrete areas are formed through the expression of specific chemokines by stromal cells within each area. CXCR4 is responsible for retaining a subpopulation of GC B cells in the dark zone and for the segregation of GC dark and light zone. In fact, CXCL12 is expressed at higher levels in the dark zone [77]. CXCR4 expression alone is insufficient to demarcate clearly distinct populations of GC B cells by flow cytometry [77] and, furthermore, it is not ideal in immunohistochemistry because chemokines receptors have a fast turnover between citoplasmatic and surface expression [78]. To perform morphologic analysis CXCR4 can be used together with other two surface molecules, CD83 and CD86, that, when combined with CXCR4, can distinguish between light and dark zone by flow cytometry: light zone B cells are CXCR4lo CD83hi CD86hi, whereas in the dark zone there are CXCR4hi CD83lo CD86lo B cells [51].

Regulation of light zone is less clear. In this compartment FDC express CXCL13. However, the difference in expression of CXCR5 between light and dark zone is minimal [51]. Mice lacking CXCL13 or CXCR5 show defects in GC size and position but light and dark zone are distinguishable [78] indicating that the two molecules are not essential for GC segregation. The GC reaction and affinity maturation, also proceeds with little alteration in the absence of either CXCR4 or CXCR5 [79, 80]. This suggests that the interplay of chemoattractants and their receptors in the GC may not be an essential requirement for selection and affinity maturation of GC B cells.

When CXCR4 and CXCR7 are expressed together, CXCR4/CXCR7 heterodimers are formed with the same efficiency as homodimers [81] and, in the presence of the chemokines, CXCR7 acts modulating CXCR4-signaling through heterodimerization sequestering CXCL12. In the lymph node CXCR7 is strongly expressed on T cells and
weakly expressed on B cells, and maybe it is involved in temporally directing B cells to the surface with the T cell zone, while CXCR4 maintains B cells in the correct position into the GC. CXCR7 functions as a scavenger and allow B cells, which continue to express CXCR4, to leave the CXCL12-rich ambience of the GC.

Differentiation of a B cell to a PC is also accompanied by a coordinated change in chemokines receptors expression. CXCR5 and CXCR7 are down-regulated, resulting in loss of responsiveness to the B and T cell zones expressing CXCL13, CCL19 and CCL21 [82]. CXCR4 is up-regulated because its expression is required for B cells homing into the CXCL12 expressing bone marrow, the major site of antibody production in adult life [83]. In line with their prominent function in the biology of B cell homing, the chemokines receptors CXCR4 and CXCR5 are widely expressed in B cell neoplasms including DLBCL [84] having a role in chronic inflammation with lymphoid neogenesis and selective homing of malignant B cells to these sites.

2.3. PLASMA CELL DIFFERENTIATION AND REGULATION

2.3.1. GC REACTIONS

During all the passages of mutation and selection, B cells differentiate into PC, and the whole process is tightly regulated by a complex network of transcription factors [85]. Gene-expression profiles of activated B cells are considerably different from those of PCs. Many of stage-specific transcription factors negatively regulate expression of genes required for following or previous stage, leading to the establishment of mutually exclusive gene-expression programmes. Figure 7 shows a summary of the main reactions occur in the GC.

PAX5 (paired box protein 5) is essential to establish and maintain the identity of mature B cells, including naïve, GC and memory B cells [86]. PAX5 regulates the expression of several genes important in GC such as CD79α (Mb1), CD19, BLNK, BCL2L1, IRF8, BACH2, SPI-B and IKZF3 (Aiolos) [87]. PAX5 also represses the transcription of genes not usually associated with B cells such as NOTCH1, CCR2 and CD28 [87]. Other PAX5 targets include genes involved in cell-cycle regulation, protein trafficking, cytoskeleton, and metabolic pathways [87]. Thus PAX5 regulates many aspects of B cell development and function, and its expression is maintained in B cells throughout all the stages [88]. PAX5 is silenced in PC since it represses different genes involved in PC differentiation, including
XBP1 and PRDM1, the gene encoding B lymphocyte-induced maturation protein 1 (BLIMP1), the master regulator of PC differentiation. PAX5 expression is suppressed by BLIMP1 to allow the beginning of PC differentiation but how is still unclear.

Figure 7: GC reactions. Many factors are required to establish and maintain the identity and function of the GC B cell, including BCL6, PAX5, SPI-B, OBF1, OCT2, BACH2, MTA3 and MITF. Red lines with stop bars indicate that a regulatory factor inhibits the indicated gene or cellular function, blue arrows indicate positive regulation. In concert, all these factors inhibit BLIMP1 expression and plasmacytic differentiation. Within the GC, the rapidly proliferating centroblasts are prone to cell death. Periodically, centroblasts move to the light zone rich in follicular dendritic cells (FDC) and follicular helper T cells (TFH), where they become centrocytes. Here, centrocytes can escape to cell death as a result of stimulation by antigen on FDC and CD40 ligand on TFH. If this does not happen centrocytes undergo to cell death.

PAX5-deficient mice underline the absolute dependence of B cell development on PAX5 [89]. In the absence of PAX5, the developmental arrest occurs at a pro-B cell-like stage. B cell progenitors lacking PAX5 completed Ig heavy chain gene rearrangements inefficiently and lacked light-chain gene rearrangements [89]. B cell progenitors lacking PAX5 are able to differentiate into other hematopoietic lineages including myeloid cells and macrophages, granulocytes, osteoclasts, NK cells, and T cells [89]. The molecular basis of this failure in lineage commitment derives from PAX5 ability to repress genes and signaling pathways, such as NOTCH1, required for development of other lineages [89]. Clearly, PAX5 plays important roles in limiting developmental potential, but the full extent of PAX5 contribution to generating B cell identity has yet to be defined.
Microphthalmia-associated transcription factor (MITF) also inhibits PC development, maintaining mature B cells in a resting state; in its absence, expression of BLIMP1 is induced, and PC form spontaneously [90]. MITF negatively regulates expression of IFN regulatory factor (IRF4), a key regulator of PC differentiation that repressed BCL6 expression and up-regulates the expression of BLIMP1.

BCL6 gene encodes a transcriptional repressor restrictively expressed at the GC stage. Here, BCL6 acts as a “master regulator” of the GC reaction and mice lacking BCL6 gene cannot generate GCs. BCL6 has different functions within established GC, promoting cell cycle progression, inhibiting differentiation, DNA-damage response and apoptosis. BCL6 is a direct repressor of BLIMP1 allowing GC reactions to continue for a sufficient duration before PC differentiation occurs [91-93]. The regulation of BLIMP1 by BCL6 requires the presence of MTA3 corepressor [91]. When MTA3 and BCL6 are over-expressed by PC cell lines, the levels of BLIMP1 and XBP1 are reduced, whereas the GC factors BLNK, SYK, CD19 and MHC class II transactivator (CIITA) are re-expressed [94]. In the absence of MTA3 expression, BCL6 expression is maintained, but expression of BLIMP1 is induced, indicating a crucial role of MTA3 in preventing differentiation into PCs. In GC, BCL6 is important also for maintaining the repression of genes involved in responses to DNA damage, and apoptosis [95, 96]. This activity of BCL6 requires its association with N-CoR, SMRT or B-CoR, together forming transcriptional repressors of genes including ATR (Ataxia telangectasia and Rad3 related). However, suppression of DNA-damage response is not absolute since if DNA damage exceeds a certain level, ATM (Ataxia telangectasia mutated) is activated inhibiting BCL6 by proteasomal degradation and thus restoring DNA damage response [97]. Moreover, BCL6 inhibits the activation of anti-apoptotic pathway by the suppression of BCL2 through Miz-1 co-factor. In this way, B cells with low affinity for the Ag can undergo to apoptosis very fast [98]. BCL6 is frequently translocated or mutated in DLBCL (30-40%) and FL (5-10%) cases. In vivo experiments with mice engineered to over-express BCL6 mimicking a chromosomal translocation found in DLBCL patients, revealed the genesis of a lymphoproliferative syndrome with features of human DLBCL [99]. BCL6 is normally regulated by different mechanisms of action including the negative transcriptional and post-transcriptional modulation by the DNA damage-driven BCL6 phosphorylation and degradation, BCL6 inactivation by acetylation and by CD40 and B cell receptor (BCR) signaling pathways. Normally, the combined BCR-CD40-triggering during late phase of GC B cell maturation extinguishes the expression of BCL6 in centrocytes allowing damaged B cells to undergo cell death. This is dependent on NF-κB signalling downstream of the CD40 receptor, that leads to the rapid ejection of N-CoR corepressor from the BCL6 repression complex associated with the ATR promoter. CD40
stimulation also leads to BCL6 repression by the induction of IRF4 that directly represses the expression of BCL6 and has been suggested to act upstream of or in parallel to BLIMP1 [100, 101].

BCL6 expression is known to be activated by IRF8 [102] and STAT3 (signal transducer and activator of transcription 3), which are present in the majority of GC B cells [102, 103], IRF8 is one of the most important transcription factors that contribute to lymphoid-lineage priming while STAT3 is involved in PC differentiation and is activated by IL21 produced by TFH cells in GC. BCL6 is also a repressor of STAT3 expression preventing STAT3-dependent induction of BLIMP1 expression. This implies a negative feedback loop and STAT3 may initiate but not maintain BCL6 expression.

BACH2 (BTB and CNC homology 1, basic leucine-zipper transcription factor 2) is another factor required for GC B cells. B cells in which BACH2 is down-regulated are not able to form GCs and do not up-regulate AID, and their Ig genes do not undergo to SHM [104]. BACH2 expression is positively controlled by PAX5 [87]. One crucial target of BACH2 in B cells is BLIMP1 [105]. This regulation could explain the effect on SHM. BLIMP1 silences AID, and its premature expression may increase PC differentiation process and decrease CSR and SHM. Indeed, CSR can be rescued in BACH2−/− B cells by inactivating BLIMP1 [106]. Thus, BACH2 is a crucial component of the genetic network controlling the timing of PC differentiation.

OCT2 and its co-activator, OBF1 are additional critical regulators of the GC response. OBF1 plays two different roles in GC processes [107]. In response to T cell dependent stimulation, OBF1−/− B cells behave similarly to BACH2−/− B cells, with a premature up-regulation of BLIMP1 and its surface marker Syndecan1 (CD138), suggesting a premature commitment to the terminal differentiation pathway. This function of OBF1 is restricted to T cell dependent cytokine stimulation, as OBF1-deficient B cells respond normally in vitro to the TLR4 lipopolysaccharide (LPS) ligand [107]. OCT2, in contrast, is more specifically required for the IL5-mediated induction of BLIMP1 and PC differentiation, through direct activation of the gene encoding the IL-5R chain [108]. Thus, OCT2 and OBF1 have both common and independent functions during B cell terminal differentiation.

In this process also proteins belonging to ETS-family of transcription factors play important roles. The ETS family of transcription factors is so named because the first gene was found in the E26 avian retrovirus. These factors share a common 85-amino-acid DNA-binding domain, which interacts with nucleotide specific sequences called ETS-binding sites (EBS), found in the promoters of their target genes [109]. Spleen focus-forming virus integration site (SPI-B) is one ETS factor involved in B cell development. SPI-B is essential for GC function reactions, required for BCR and T cell dependent antibody responses [44].
Cells lacking SPI-B activity fail to proliferate in response to IgM cross-linking, exhibit limited capacity to respond to T cell dependent antigens, and produce low levels of IgG1, IgG2, and IgG2b [110]. SPIB-deficient mice also show immunological disorders with GCs smaller in size, and more apoptotic cells than those in wild type animals. SPI-B protein levels are high in naïve, memory and activated human tonsil B cells, but undetectable in human PCs and, in line with this, enforced expression of this factor in human B cells directly repress both BLIMP1 and XBP1 gene expression blocking PC differentiation and Ab production [111].

Other two ETS factors, ETS1 and FLI1, are described having a role in GC B cell development, which I will present in a more extended way, representing the main topic of my thesis.

**2.3.1.1. ETS1, v-ets avian erythroblastosis virus E26 oncogene homolog 1**

ETS1, the founding member of the ETS family, is located on chromosome 11 and is expressed in a variety of cells, including B and T cells, endothelial cells, vascular smooth muscle cells, epithelial cells as well as neoplastic cells.

The major isoform of ETS1 in human is encoded by 8 exons forming the full length p54 (p51 based on predicted molecular weight) composed by 441 amino acids. The ETS1 protein is characterized by a well-conserved winged helix-turn-helix DNA binding domain (DBD) (Fig.8), with which it binds to the specific EBSs on the promoters of its targets genes. The DNA binding domain is flanked by two autoinhibitory domains composed by two alpha helices, HI-1 and HI-2, and a serin rich region located in the N-terminal part of the ETS domain (exon VII), and by two other alpha helices H4 and H5 in the C-terminal region of the ETS domain. These two regions interact with each other creating an autoinhibitory conformation that prevents the DNA binding. Serines within the exon VII could be phosphorilated by calmodulin kinase (CAM) in a calcium-dependent manner and this phosphorylation cause a decreased in the flexibility that characterized the active DNA binding form of ETS1 leading to a more rigid conformation of the ETS domain and to a progressive reduction in DNA binding activity. Exon VII is absent in the shorter isoform p42 of 356 amino acids long making that isoform more active in binding the DNA than the full length p54 (Fig.8).

ETS domain is also involved in protein-protein interaction allowed by a conserved PNT (or Pointed) domain within ETS1 protein (Fig.8). PNT is structurally related to the larger group of sterile alpha motif (SAM) domains through a common tertiary arrangement of four alpha helices. SAM domains are typically found in numerous proteins involved in eukaryotic developmental and signal transduction pathways. PNT domain is a docking
site for mitogen–activated protein (MAP) kinases such as ERK2 that phosphorylate ETS1 at a conserve threonine residue (T38) close to PNT domain leading to a strong increase in the transcriptional activity of ETS1 mediated by CREB binding protein (CBP) recruitment.

Finally, ETS1 harbors an acidic transactivation domain (TAD) that is essential to activate transcription activity of ETS1. TAD is required for the interaction with CBP and p300 that function as histone acetyl transferases and co-factors.

A third isoform of 225 amino acid long, p27 (Fig. 8), arises from splicing out of the threonine-38 residue, the PNT and TAD domains but retains the DNA binding and both the autoinhibitory domains surrounding that domain thus p27 isoform can bind and autoinhibit its binding to the DNA but it cannot transactivates its target genes.

In addition to phosphorylation, ETS1 protein structure can be modify by sumoylation and ubiquitinylination [354,355]. Small ubiquitin-like modifier (or SUMO) proteins are a family of small proteins that modify the function of proteins that bind. Sumoylation is directed by an enzymatic cascade analogous to that involved in ubiquitinylination but in contrast to ubiquitinylination, sumoylation is not use to tag proteins for proteasomal degradation. In fact, SUMO modification appears to play a role in a variety of cellular processes including protein–protein interaction, subcellular localization, protein stabilization and transcriptional regulation [356]. The major sumoylated sites of ETS1 are lysine 15 and lysine 227, and UBC9 (E2 conjugated enzyme) and PIASy (E3 lygase) are the two proteins involved in sumoylating ETS1 [355, 357]. This post-transcriptional modification does not affect the nuclear localization of ETS1 but inhibits its activity in transactivating genes and could be reverted with the help of the SUMO protease SEMP1.

Moreover, ETS1 is polyubiquitinylated on the lysine 48 residue independently on sumoylation. These modifications appear regulate ETS1 activity in a different manner, with polyubiquitinylation regulating its protein stability and sumoylation reducing its transcriptional activity [354].

ETS1 is important in various biological processes such as development, differentiation, proliferation, apoptosis, migration and tissue remodelling. ETS1 acts as an oncogene that controls invasive and angiogenic behaviour of malignant cells in multiple human cancers [113, 114]. ETS1 has been implicated in the activation of metastasis-associated molecules and its expression in ovarian carcinoma is associated with poor prognosis [115]. ETS1 has also a role in human breast cancer [116], lung cancer, colorectal and squamous cell carcinoma [117], ETS1 expression is associated with a higher incidence of lymph node metastasis [118-120] while in prostate cancer its expression is associated with a progressive disease [121]. ETS1 expression increases upon treatment with angiogenic
factors such as tumor necrosis factor 1 (TNF1), phorbol myristate acetate, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). ETS1 binding sites have been identified in numerous promoters of genes that are involved in angiogenesis, including the VEGF receptors, FLT1 and FLK1. Overexpression of ETS1 results in cellular transformation in vitro and fibroblasts expressing high levels of exogenous ETS1 gene product are tumorigenic in vivo [122, 123].

ETS1 has an important role in immunity. In the absence of ETS1, T cell numbers are reduced due to impaired survival and ability to respond to proliferative stimuli. A few T cell-specific genes are regulated by ETS1. The enhancer of the gene encoding the α chain of the TCR contains an EBS specific for ETS1 [124]. TCRβ is regulated by ETS1 in a negative manner. Immature T cells without ETS1 coexpress TCRs with different TCRβ chains, indicating that ETS1 is involved in allelic exclusion [125]. The ETS1 levels are high in resting T cells and are reduced after activation suggesting that ETS1 is important for the quiescence of T cells [126]. However, even if the levels of ETS1 are low in activated T cells, ETS1 is involved in the transactivation of the gene encoding granulocyte-macrophage colony-stimulation factor (GM-CSF) following T cell activation, and this activity is repressed when Calmodulin-dependent kinase II (CaMKII) phosphorylates ETS1 protein. GM-CSF is a cytokine that regulates the growth and differentiation of dendritic cells, myeloid progenitors, and granulocytes and is involved in inflammatory and autoimmune diseases [127]. T cells and macrophages produce the majority of GM-CSF. ETS1 have also demonstrated ability in regulating the activity of IL-5 promoter in activated T cells and myeloid cells together with the ETS1 closely related factor ETS2 and GATA3 [128]. Depending on the presence of specific cofactors, several ETS factors have this ability to act as either an activator or a repressor of transcription. In contrast to IL-5, also IL-2, important for B and T
cell proliferation and enhanced B cell Ig secretion, in particular IgM [129], is regulated by ETS1 but in a negative manner [130].

Regarding its role in B cell development, ETS1 is highly expressed in naïve B cells with decreased expression following B cell activation and terminal differentiation. ETS1 has been shown to cooperate with PAX5 in gene target activation. In particular ETS1 forms a ternary complex on the CD79α promoter (mb-1 gene, encoding for the immunoglobulin-associated alpha chain, Igα) with PAX5 and FLI1 [131]. ETS1 also leads to PAX5 up-regulation in B cells [132], and might contribute to the regulation of plasmacytic differentiation. Upon TLR9 stimulation, in vitro ETS1−/− B cells differentiate into IgM-secreting PCs, suggesting that ETS1 functions to limit TLR9 signalling pathway and thereby limiting B cell terminal differentiation [132]. Mice with a deletion of the Pointed domain of ETS1 gene and expressing low levels of an ETS1 protein incapable of being functionally activated exhibit an increased number of IgM-secreting PCs [133]. These mice possess very few marginal zone B cells (CD20hi CD23lo) and have an increased expression of activation markers on follicular B cells (CD20lo CD23hi). These observations support the idea that ETS1 is a critical negative regulator of B cell terminal differentiation induced by TLR9, and that ETS1-deficient B cells have an intrinsic propensity to undergo differentiation into PCs, confirming the important role that ETS1 plays during B cell developmental process. ETS1 physically interacts with BLIMP1, leading to a block of BLIMP1-mediated repression of its target genes without interfering with BLIMP1 levels [132].

2.3.1.2. FLI1, friend leukemia insertion 1

FLI1 is an ETS family member located close to ETS1 gene on chromosome 11. FLI1 was originally identified as a proto-oncogene as a site for retroviral integration of Friend virus-induced erythroleukemias.

The FLI1 gene encodes two isoforms of 51 (canonical transcript) and 48 kDa through the use of two alternative and highly conserved in-frame initiation codons, AUG +1 (p51) and AUG +100 (p48) (Fig.9) [134]. Both isoforms differ in their N-terminal end but retain the same functional domains and activity [134]. Two different promoters regulate the transcription of the two splice variants but several transcription factors binding sites, such as sites recognized by CREB, E2A, Oct-3, Sp-1 and c-Myc, are maintained inside both promoter sequence. The promoter encoding for the 48 kDa isoform of FLI1 has very strong transcriptional activity compared to the canonical FLI1 isoform [358].

Moreover, both FLI1 isoforms are cleaved by caspase 3 [359]. In fact, FLI1 has three different and conserved aspartate residues (D20, D155 and D209) involve in the caspase 3 cleavage activity, but maybe the D155 residue is not cleaved in vivo [359].
different products are generated by the longer isoform, while only two products arise from the shorter one and this is because the first site of cleavage is inside the N-terminal end specific for the 51 KDa FLI1 isoform [359]. This regulation suggests that FLI1 is a potent anti-apoptotic agent and that its cleavage is functionally important in vivo.

FLI1 functional domains include the pointed domain and a FLI1-specific region (FLS) referred to as the amino terminal transcriptional activation (ATA) domain in the amino terminal half of the protein, an ETS domain and finally a carboxy terminal transcriptional activation (CTA) domain (Fig.9). The ETS domain is responsible for DNA binding activity having the winged helix structure typical of ETS family members and recognizes the specific sequence ACCGGAAAG/AT/C. The CTA domain is involved in transcriptional activation and protein-protein interaction such as ATA domain but it can simultaneously acts as a transcriptional activator and repressor, while ATA domain is used only for transcriptional activation. The ETS domain contains sequences of secondary structures like helix-loop-helix (H-L-H) and is homologous to the DBD domain of ETS1. Helix-loop-helix structures are also identified in the ATA domain. The FLS and CTA domains contain sequences, which resemble turn-loop-turn (T-L-T) secondary structures. All these structures suggest that FLI1 acts as a transcriptional regulator interacting with others proteins.

FLI1 is well known as an oncogene and is expressed transiently during embryogenesis, while in adult is expressed highly in hematopoietic tissue and endothelial cells with lower levels detect in lung, heart and ovaries [135]. FLI1 is known to take part in vasculogenesis, differentiation of megakaryocytes, promotes cell cycle and inhibits apoptosis [114]. FLI1 is able to down-regulate Rb protein expression leading to the transition of cells through the S phase [136], while its role in apoptotic inhibition correlates with up-regulation of BCL2 expression [137]. FLI1 roles in vasculogenesis and in inhibition of collagen biosynthesis have an impact in systemic sclerosis or scleroderma (SSc), in which reduced levels of FLI1 in endothelial cells may play a critical role in the development of SSc vasculopathy. FLI1 has also been reported to inhibit erythroid differentiation. Together with PU.1, another ETS family member, FLI1 may inhibit erythroid differentiation through functional interference between these ETS family proteins and nuclear hormone receptors [138].

FLI1 gene is rearranged in 95% of Ewing’s sarcoma (ES), a pediatric tumor with neuroectodermal origin [139]. The translocation t(22;11) occurs between the central exons of EWSR1 on chromosome 22 and to the central exons of FLI1 creating a fusion protein acting as a transcriptional activator that recognizes the EBS specific for FLI1. The fusion proteins possess increased transactivation potential in comparison with the wild type FLI1 and this activity is thought to contribute to malignant transformation of the cells
Indeed, the fusion protein, which shows stronger transactivation potential than the former, is often associated with poor prognosis [141]. Overexpression of EWS-FLI1, but not wild type FLI1, transforms NIH/3T3 cells. The expression of EWS-FLI1 leads to a strong up-regulation of the c-myc oncogene [142]. Tumorigenic role of EWS-FLI1 fusion protein is also promoted by the inhibition of p53 activity. EWS-FLI1 fusion protein, in fact, inhibits the p300-mediated acetylation of p53 at Lys-382 suppressing its transcriptional activity and enhancing MDM2-mediated p53 degradation [143].

**Figure 2**: FLI1 splice isoforms with functional domains. The full length of FLI1 proteins (p51) consist of 452 amino acids which contain the following domains: ATA: amino-terminal transcriptional activation domain, FLS: FLI1 specific domain, CTA: carboxy-terminal transcriptional activation domain. The functional domains are the same in the shorter FLI1 isoform p48. D20, D155 and D209, aspartate residues.

FLI1 has also an important role in the regulation of autoimmunity. Overexpression of FLI1 protein in transgenic mice results in the development of a lupus-like disease, including hypergammaglobulinemia, splenomegaly, B cell peripheral lymphocytosis, progressive immune complex-mediated renal disease and ultimately premature death from renal failure [144]. Reduced expression of the FLI1 protein in MRL/lpr mice, a murine model of lupus, significantly increases survival and decreases renal disease compared with wild type [145]. In addition, MRL/lpr mice also had decreased total serum Ig. FLI1 expression correlates with patients disease activity, meaning patients with highest disease activity express the highest FLI1 levels and vice versa [146].

In B cells, FLI1 has been reported to coregulate Igα expression with PAX5. Mice with reduced levels of FLI1 have reduced Igα expression and this reduction may contribute to a decreased BCR signaling and concomitant decreased number of follicular B cells and increased number of marginal zone B cells [147]. Reduced mRNA levels of Igα after FLI1 decreased expression is accompanied by a reduction in E2A and ERG1 expression and an increased in Id1 and Id2 expression, all known to be regulators of B cell development. Proliferation of B cells is reduced although intracellular Ca²⁺ flux in B cells from mice with
reduce quantities of FLI1 is similar to that of wild type controls after anti-IgM stimulation. Finally, immune responses and in vitro class switch recombination are altered in FLI1-deficient mice. Taken together, these studies suggest that FLI1, such as ETS1, plays an important role in the immune system including B cell.

2.3.2. PLASMA CELL DIFFERENTIATION

Once a GC B cell undergoes different cycles of mutations and selection for affinity maturation, it is ready to differentiate into PC and to produce Abs against a specific antigen. At this point a drastic change in expression of transcription factors is needed (Fig.10). Several transcription factors must be up-regulated such as BLIMP1 and XBP1 or down-regulated such as BCL6 and PAX5 [148, 149].

In this process, the BCR plays a key role, and the strength of the interaction with the antigen is critical in determining which B cells enter PC differentiation. In fact, the ligation of the BCR and the CD40 molecule leads to the expression of IRF4, downstream to NF-kB activation that inhibits BCL6 transcription and promotes the expression of PRDM1 but only after a strong signal from BCR with the help from cytokines or interleukins (IL-4, IL-5 or IL-2) [150]. B cells express distinct amounts of IRF4 during the different phases of development. Naïve resting B cells express a low basal amount of IRF4 that is rapidly increased during the maturation process [100]. However, it is not clear whether IRF4 alone is sufficient to induce expression of BLIMP1 and trigger PC differentiation. BCR activation stimulates also the MAPK cascade that leads to BCL6 inhibition by phosphorylation and subsequently proteasomal degradation, linking BCL6 turnover with BCR stimulation [151]. BCL6 as said before, is the master regulator of GC reactions and its repression is essential because allows the activation of BLIMP1 expression. BLIMP1, encoded by the PRDM1 gene, is the master regulator of PC differentiation and function as a transcriptional repressor. Within the B cell lineage BLIMP1 starts to be expressed in centrocytes [152] and is maintained in all antibody secreting cells (ASCs) [153], with cycling plasmablasts being distinguishable from long-lived PCs based on their expression of BLIMP1 in both mouse [153] and human [154]. Once BLIMP1 is activated it represses many important genes of GC B cell program in addition to BCL6 and PAX5, including genes encoding SPI-B, Id3, CIITA and MYC [111, 155, 156] thereby ensuring that, after PC development is induced, B cells cannot return to an earlier developmental stage. In particular, BLIMP1 expression is mutually exclusive with BCL6 and PAX5 expression ensuring that the phases of B cell maturation remained completely distinct.
Introduction – Development of mature B Cells

Figure 10: interaction between centrocytes with FDC and TFH by BCR and CD40 respectively, allowed that PC differentiation take place. Once expressed, PC factors repress factors required for GC formation and B cell identity. NF-kB is activated after the stimulation by CD40 molecule on TFH. NF-kB activates the expression of IRF4 that initiates plasmacytic differentiation by establishing a characteristic regulatory network, which extinguishes the mature B cell program while promoting terminal PC differentiation and antibody production through XBP1. IL-21 secretion by TFH induces BLIMP1 in a STAT3-dependent manner while BCR stimulation by Ag on FDC inhibits BCL6 by inducing phosphorylation and degradation of it. Ca^{2+}-loaded Calmodulin (CaM) is also triggered by BCR activation leading to GC genes down-regulation through E2A inhibition. Red lines with stop bars indicate inhibitory regulations blue arrows indicate activating regulations. Red wide arrow indicates down-regulation of all the alongside genes. Blue wide arrow indicates up-regulation of the alongside gene.

Hauser J. et al. [155] have tried to understand which genes trigger the switch into PC phenotype and the timeline of expression changes. From this study the primary regulatory events identified were inhibition of BCL6 as expected, together with PAX5, MITF, ETS1, FLI1 and SPI-B known as direct or indirect BLIMP1 repressors. These are very rapid events that occur within 30 minutes after BCR, CD40 or TLR stimulations [155]. Ca^{2+}-signalling activated by BCR stimulation leads to inhibition of E2A by Ca^{2+}-loaded Calmodulin (CaM) followed by BCL6, PAX5, ETS1 and FLI1 expression changes, with a weak increment of BLIMP1 [155]. BCR stimulation increases IRF4 and BLIMP1 mRNA levels relatively fast, but slightly slower than the rapid transcriptional down-regulations and this is
because IRF4 and BLIMP1 are more dependent on NF-κB activation than Ca\textsuperscript{2+}-loaded Calmodulin (CaM) pathway \cite{155}. The genes down-regulated through Ca\textsuperscript{2+}-loaded Calmodulin (CaM) pathway could all be direct targets of E2A. This transcription factor functions in hematopoietic progenitor cells to activate expression of PAX5 and establishment of B cell specific gene expression program and when its expression is inhibited, BCR activation has no effect on the expression of any of previously described GC genes \cite{155}.

Besides repressing important GC transcription factors, BLIMP1 up-regulates genes involved in Ig secretion such as Ig genes themselves (IgH, IgL and the J chain) and X-box binding protein 1 (XBP1), a transcription factor essential for antibody secretion \cite{149}. XBP1 is up regulated as part of the endoplasmic reticulum (ER) stress response and is expressed at a high level in PCs where is essential for inducing the secretory phenotype. This gene is a mediator of unfolded protein response (UPR) activated by accumulation of misfolded protein in the ER \cite{157}. XBP1 activity is further regulated by a splicing event that is also induced by the UPR \cite{158}, forming the splice active form XBP1s that present an excised 26-bp intron, causing a frameshift producing a new C-terminus that confers transcription activating activity \cite{159}. XBP1 is the only transcription factor that is uniquely required for PC differentiation but it is not, apparently, required for any early stage of B cell development. In mature B cells, XBP1 is maintained at low levels thanks to PAX5 activity and the activation of BLIMP1 factor represses this regulation by repressing PAX5 \cite{160}. The mutually exclusivity in their expression ensures that the phases of B cell maturation remained completely distinct.

2.4. REMARKS

The GCs are the histological structures where B cells undergo genetic remodeling of their Ig genes toward the production and selection of cells able to recognize the antigens with high affinity. As mentioned above, almost all B-NHL derived from GC B cell at different stages of differentiation maintaining several molecular and phenotypic characteristics of the normal B cell counterparts. Thus is very important to clarify molecular mechanisms governing normal GC and post-GC processes. A large number of studies have identified several transcription factors that have to be up-regulated or down-regulated during B cell differentiation. However, a lot of questions about the role of several genes whose expression is regulated during B cell differentiation are still opened. In particular, ETS1 and FLI1 are described as GC players that must be silenced to allow
BLIMP1 up-regulation and PC differentiation. But, what is their specific role in GC? It is also known that ETS1 and FLI1 interact with other important GC transcription factors such as PAX5 and that ETS1 lack lead to a propensity in PC differentiation under TLR9 stimuli, while the lack of FLI1 leads to the onset of autoimmune disease. But, how ETS1 and FLI1 contribute in the regulation of PC differentiation? What are their direct targets? These are some questions to which I will try to answer.
3. Genetics of Diffuse Large B Cell Lymphoma
Although the GC response is essential for a proper immune response, it come with a risk. As we already mentioned, in GC, two main mechanisms of genetic lesions occur that could generate lymphomas: chromosomal translocations and aberrant somatic hypermutations. Both are products of AID-dependent DNA remodelling that characterize GC B cells. These physiologic mechanisms of DNA remodelling required during the GC reaction could be hijacked by lymphomagenesis. These genetic abnormal alterations and copy number changes of specific chromosomal regions contribute to the tumor pathogenesis of DLBCL. Based on gene expression profiling analysis, three main biologically and prognostically significant subgroups have been recognized: germinal centre B cell-like (GCB) DLBCL, activated B cell-like (ABC) DLBCL, primary mediastinal B cell lymphoma (PMBL) [14, 161] and, integrating morphology, genetics and clinical feature, a series of DLBCL variants are recognized in the 2008 World Health Organization lymphoma (WHO) classification. The three DLBCL subgroups, deriving from different stages of GC differentiation, present quite specific pattern of underlying genetic lesions that I will now summarize.

### 3.1. Germinal Centre B Cell-like Diffuse Large B Cell Lymphoma

The gene expression pattern of GCB-DLBCL is very similar to that of normal GC B cell retaining its typical features. Many aberrations detected in GCB-DLBCL involve the pathways that are tightly regulated during the specific stages of GC development. When these processes cease to be properly regulated GC B cells can block their differentiation and undergo an uncontrolled growth.

### 3.1.1. BCL2 Chromosomal Translocation

The anti-apoptotic oncogene BCL2 (18q21) is very often deregulated in DLBCL by a whole array of mechanisms. The chromosomal translocation t(14;18)(q32;q21) juxtaposes the BCL2 gene to the immunoglobulin heavy chain (IGHV) gene enhancer (14q32) resulting in a deregulated expression of the gene, also disrupting suppression by BCL6 [162]. The t(14;18) is the most common translocation in GCB-DLBCL, detected in 30-40% of the cases [19, 163]. The translocation is not specific of DLBCL, being present in over 90% of follicular lymphoma (FL), and, indeed, some of the GCB-DLBCL might represent
transformation from clinically silent FL [164]. t(14;18)-positive circulating B cells can be detected in the blood of 50%–70% of healthy individuals who are not prone to developing FL, and the incidence of FL and the frequency of t(14;18)-positive cells in healthy individuals increases with age. Most of these cells are not naive B cells, but instead are IgD+CD27+ (or IgM+CD27+) memory cells. These data suggest that translocation of BCL2 is insufficient to induce lymphomagenesis but other genetic events are necessarily to generate FL or the most malignant DLBCL.

The t(14;18) is virtually absent in cases of GCB-DLBCL diagnosed at an age of less than 18 years [165], and less common in cases of the Waldeyer’s ring [166]. Although rarely translocated, BCL2 is much more recurrently gained or amplified in ABC-DLBCL (30-40% of the cases) than in GCB-DLBCL (15%) [126, 161].

The clinical relevance of the presence of the t(14;18) itself and of the expression of the BCL2 protein has always been controversial [126, 167, 168]. Although the prognostic impact likely depends on the type of treatment received by the patients (for example, R-CHOP versus CHOP), there are certainly technical biases that make difficult to draw any strong conclusions. Two different large collaborative groups have recently analyzed the prognostic impact of the translocation and of the protein expression in R-CHOP treated patients [18, 126]. Only one of the studies associated the presence of the t(14;18) with a poor outcome in GCB-DLBCL [18]. Both studies identified BCL2 protein as a poor prognostic marker in GCB-DLBCL and not in ABC-DLBCL, in contrast with what previously reported in CHOP-treated patients in whom BCL2 expression was a poor prognostic marker only in ABC-DLBCL [169].

BCL2 gene and its promoter region are also very commonly mutated [162, 170-172]. Likely the effect of aberrant somatic hyper mutations (ASHM), BCL2 mutations are observed almost exclusively in GCB-DLBCL and are associated with the presence of the t(14;18). While mutations targeting the promoter regions cause the loss of the MIZ1-mediated BCL6 suppression of BCL2 [162], mutations affecting the coding part of the gene have been hypothesized altering the interaction of the BCL2 protein with other molecules, such as TP53 protein [170]. Despite early report [170] these mutations would not cause false negative results at immunohistochemistry.

3.1.2 MYC DEREGULATION

Chromosomal translocations deregulating MYC (8q24) occur in DLBCLs with a frequency of 5–15%, more commonly in GCB-DLBCL, among which they represent the second most common translocations [173]. MYC protein is expressed in both GCB- and ABC-DLBCL
subtypes suggesting a role of the gene independent to the COO. MYC is important for cell-cycle progression, metabolism, protein synthesis, stem-cell renewal, and mRNA regulation but can induce apoptosis by increasing p53 expression or amplifying apoptotic signaling pathways [112, 136, 139, 146].

The t(8;14)(q24;q32), deregulating MYC by juxtaposing in proximity of the IGHV enhancer [174], is the same characteristic cytogenetic event of BL but the MYC gene can also be translocated to other genomic loci and is often co-detected together with rearrangements of BCL2 or BCL6 (“double-hit” lymphomas) [27, 115, 116, 121, 175-184]. MYC translocation is associated with a very poor outcome in patients with DLBCL treated with R-CHOP [115, 116, 120, 121, 180, 184], and different papers indicate a determinant role of BCL2 translocation occurring in MYC-positive DLBCL patients [18, 185]. On the contrary, the clinical implications of extra copies of MYC gene are less clear [176, 177, 186, 187]. Immunohistochemistry protocols have become available in the last few years, able to predict cases bearing a MYC gene translocation [18, 185, 188]. Cases of both primary and secondary DLBCL with a MYC translocation reproducibly are described having >50% of tumor nuclei with a strong positivity for MYC protein [189]. Interestingly, MYC protein expression detected by immunohistochemistry correlates only with MYC gene translocation and not with MYC gene copy number gain [182]. Unfortunately, well-established strategies to modulate the function of the MYC oncoprotein do not exist yet.

In the minor part of the results, I will present a correlation analysis between MYC copy number status and the OS of DLBCL patients carry or not extra copy of MYC gene. For this analysis was used the same cohort of DLBCL patients used for the main functional analysis of this thesis. This part had the aim to assess the role of MYC gain in clinical outcome of DLBCL patients.

3.1.3 CHROMATIN REMODELLING

Chromatin remodeling is the dynamic modification of chromatin architecture that allows the access of condensed genomic DNA to transcription factors and RNA polymerase II and thereby allowing the expression of genes. The DNA is folded into nucleosomes that comprise approximately 147 bp of DNA wrapped around a histone octamer. Between histones, the histones H2A, H2B, H3 and H4 can be chemically modified, on specific amino acids residues, by chromatin regulators such as histone acetyltransferases (HATs), deacetylases (HDAC), methyltransferases (HMTs), and kinases resulting in an open or
closed chromatin state in dependence to the type of histone modification (Fig.11). The change in chromatin configuration reflects the regulation of expression of genes. Chromatin remodeling is important for cell growth, cell-cycle progression, DNA repair and chromosome segregation, and, consequently, in tumorigenesis. Mutations in chromatin regulators cause deregulation in histone modifications and potentially favor self-sufficiency in cell growth and escape from growth-regulatory cell signals, two important hallmarks of the cancer [190].

Recently, the use of deep-sequencing combined with DNA profiling has identified recurrent somatic mutations in genes encoding chromatin modifying enzymes, including HMTs and HATs [171, 191, 192]. In DLBCL, the most commonly affected genes are MLL2, CREBBP, EP300, EZH2, MEF2B. The mutations are not DLBCL specific, detected also in other lymphomas. Some of them are shared by the GCB- and the ABC-DLBCL (MLL2, CREBBP, EP300), while others are detected almost exclusively in GCB-DLBCL (EZH2, MEF2B) [19, 171, 191-193] and especially in GCB-DLBCL bearing BCL2 translocations (EZH2).

**Figure 11:** Superimposed layers of condensed chromatin are associated with inactive (OFF) transcription of genes. Chemicals modifications of specific amino acids residues on histones allow access to a gene of DNA-binding proteins such as transcription factors (TFs) and RNA polymerase II (RNAPII) and thereby activating it (ON). The DNA will then make mRNA, the blueprint for protein production. Figure adapted from www.drugabuse.org, NIDA notes 2007.
Inactivating mutations occur in the tumor suppressor gene MLL2 in up to one quarter of DLBCL [171, 192]. MLL2 codes for a histone trimethyltransferase, promoting the expression of its target genes modifying the histone 3 on lysine 4 (H3K4me3). The acetyltransferase genes CREBBP and EP300 are inactivated by somatic mutations or DNA losses in approximately 15% and 5% of DLBCL cases, respectively [19, 171, 192]. CREBBP and EP300 belong to the KAT3 family of histone/protein lysine acetyltransferases [194, 195], and regulate important cellular proteins. In particular, since acetylation of BCL6 by EP300 determines the loss of the protein transcriptional repressing activity, while TP53 acetylation is required for TP53 activation, the mutations contribute to BCL6 activation and TP53 inactivation in DLBCL [191].

MEF2B is a member of MEF2 gene family encodes for transcription factors that recruit histone-modifying enzymes including HDACs and HATs. Activating mutations of the gene are observed in 10-18% of DLBCL [171, 172, 191, 192]. MEF2B positively regulate BCL6 expression, and its mutations contribute to BCL6 deregulation of DLBCL [196].

EZH2 encodes a HMT, a member of polycomb complex PRC2, responsible for methylation of histone 3 on lysine 27 (H3K27) leading to transcriptional silencing [197]. EZH2 mutations tend to occur in association with the presence of the t(14;18), in approximately 20% of GCB-DLBCL and virtually never in ABC-DLBCL [19, 161, 193]. Mutations affect the SET domain of EZH2, in particular the tyrosine Y641 that corresponds to a key residue in the active site of the protein. The mutations lead to a change of the enzyme affinity for its substrate: while the wild type EZH2 has an affinity higher for the unmethylated H3K27 (H3K27m0), lower for dimethylated H3K27 (H3K27me2) and even lower for the trimethylated histone H3 on lysine 27 (H3K27me3), the mutant protein has the highest affinity for the H3K27me2 determining an hyper-trimethylation of H3K27 [198, 199]. Promising pre-clinical data have been presented inhibiting EZH2 activity [199-202] and early clinical trials are now running (NCT01897571).

All the mutations affecting genes coding chromatin remodelling proteins usually affect only one allele, suggesting a role for these chromatin-remodelling enzymes in haploinsufficiency or the need of the remaining wild type allele to be retained functional as in the case of EZH2 [191, 192]. Mutations affecting chromatin modifiers, for example, MLL2, CREBBP and EP300, tend to occur in a mutually exclusive way across the different samples, suggesting that alterations at these genes converge on a common transcriptional program deregulation [191]. The generalized alteration of the whole mechanism of chromatin regulation provides the rational to pharmacologically reverse the epigenetic changes induced by the genetic alterations. The already mentioned EZH2 inhibitors, histone deacetylase inhibitors (HDACi), demethylating agents and
bromodomain inhibitors represent classes of compounds that are under active preclinical and clinical development.

3.1.4 BCL6 DEREGULATION

BCL6 translocation is found in 10% of GCB-DLBCL cases [203] but the majority of BCL6 translocation cases are found in ABC-DLBCL (about 25% of the cases) [203], as we will see later. In this GCB subgroup BCL6 is found expressed at high levels and this could reflect the cell of origin, since B cells normally express BCL6 during GC phase. However, in GCB-DLBCL BCL6 gene can be mutated due to the aberrant activity of SHM mechanisms [63]. In particular, mutations affect the 5' untranslated exon 1 of the gene leading to a block in the mechanisms through which BCL6 is regulated by itself and by other transcription factors expression [63, 204]. Disruptions or mutations of these sequences inhibit the normal mechanism of regulation leading to constitutive expression of BCL6. The over-expression deregulates multiple oncogenes and tumor suppressors, including BCL2, TP53, CDKN1A, CDKN1B, CCND1 and CCND2 that are also regulators of BCL6 expression leading to a deregulated loop.

3.1.5 OTHER LESIONS

A locus on chromosome 2p (2p12-p16) is recurrently gained in GCB-DLBCL and PMBL, and only rarely in ABC-DLBCL [19, 161]. Conflicting data exist on the genes affected by the lesion. REL and BCL11A oncogenes are among the most up-regulated genes associated with this aberration. REL encodes a NF-κB transcription factor subunit. BCL11A is a zinc-finger protein that interacts with BCL6. The expression of these two factors is frequently observed in GCB-DLBCL subgroup that have favorable prognosis. REL seems more frequently gained than BCL11A [205] and this could suggest that REL might play a more important role in lymphomagenesis of GCB-DLBCL. NF-κB pathway is constitutively activated in ABC-DLBCL [206], but it seems to be relevant also in at least a subset of GCB-DLBCL [207], in which REL may be used to enhance the NF-κB response to signals from the microenvironment. NF-κB activation is pathogenetically relevant only during early phases of GCB-DLBCL development. NF-κB pathway is essentially silent in GC B cells, as measured by expression of NF-κB target genes and by nuclear accumulation of NF-κB heterodimers [208]. However, NF-κB pathway activation is periodically sustained by CD40 signaling in GC reaction and the gain of 2p12-p16 locus could enhance responsiveness to extracellular stimuli that engage the NF-κB pathway. Alternatively, other genes
mapped close to REL and BCL11A genes, namely PUS10 [161], ASHA2, MDH1 and UGP2 [209], might be involved. In the GCB-DLBCL subgroup gains and amplifications in the MIRHG1 locus (13q31.3) have been identified that cause an over-expression of the mir-17-92 microRNA polycistronic cluster. This event is documented in different B cell lymphomas and is correlated with MYC rearrangements or amplifications [210], and, also, MIRHG1 positively regulates MYC and vice-versa: indeed, cases with 13q31.3 amplification express MYC and MYC target genes at significant higher levels than cases without the abnormality [161, 186, 210-215]. Furthermore, MIRHG1 over-expression leads to an increased in cell proliferation and cell survival also inhibiting the tumor suppressor gene PTEN and the proapoptotic gene BIM [216]. Approximately 10% of GCB-DLBCL cases carry deletion of chromosome 10q containing PTEN gene, and this is mutually exclusive with the amplification of MIRHG1 gene [19, 161]. In a more recent paper [140], loss of PTEN was found in 55% of GCB-DLBL and in 14% of ABC-DLBCL analyzing a cohort of 248 primary DLBCL patient samples. In GCB DLBCL the PTEN status was inversely correlated with activation of the oncogenic PI3K/protein kinase B (AKT) pathway in both DLBCL cell lines and primary patient samples [140]. Normally, PTEN inhibits AKT that remaining active promotes cell growth, cell motility, and angiogenesis in GCB-DLBCL also acting on MYC expression activation [19, 140, 161]. Gain on chromosome 12 is observed in about 10% of samples, and possibly causing up-regulation of MDM2 gene encoding a negative regulator of the tumor suppressor gene TP53, is much more common in GCB-DLBCL than in ABC-DLBCL [19, 161].

Gains affecting chromosome 7, largely in its integrity, are more frequent in the GCB-subtype. Its pathogenetic effect is still unclear, but it might cause an over-expression of a series of miRNA mapped on the chromosome, especially MIR96, MIR182, MIR589, MIR25 [217]. The presence of 7q gains seems to predict a better outcome and a low probability of bone marrow involvement [217, 218].
3.2 ACTIVATED B CELL-LIKE DIFFUSE LARGE B CELL LYMPHOMA

Lesions described more commonly in ABC-DLBC than in GCB-DLBC act mainly via inducing a block in the late GC-phase pre-PC stages and via determining a constitutive activation of the BCR signaling and NF-kB pathway.

3.2.1 B CELL DIFFERENTIATION

In ABC-DLBC, BCL6 (3q27) is frequently affected by chromosomal translocations (25% of cases) [219] and by somatic mutations in its regulatory regions. All these lesions are believed to cause the deregulation of BCL6 expression by preventing its silencing at the conclusion of the GC response.

BCL6 controls different cellular functions, including DNA damage responses, cell cycle progression and signal transduction [11, 65, 91, 162, 203, 204, 220-222]. BCL6 works also by repressing miRNAs, such as miR-155, which negatively affect the expression level of important GC genes (for example, AID, SPI1, IRF8 and MYB) [223]. BCL6 expression has been associated to a better outcome, likely reflecting the GCB signature, without a correlation with BCL6 translocations or BCL6-deregulating mutations [224].

Frequent deletions found in DLBCL affect various regions of the long arm of chromosome 6, mainly 6q23 (containing TNFAIP3) and 6q21 (containing PRDM1/BLIMP1) [192, 207, 222, 225-227]. Frequent structural PRDM1 alterations, as well as somatic mutations, are almost exclusive of ABC-DLBC, which all determine an impairment of PRDM1 trans-repression activity. BCL6 is one of the most important direct repressor of BLIMP1 expression, and BCL6 alterations represent an alternative mechanism of BLIMP1 deregulation. Notably, genomic events targeting BCL6 (translocations or mutations in its regulatory regions) are mutually exclusive with PRDM1 mutations/deletions [191, 226]. These observations suggest BCL6 deregulation and PRDM1 inactivation represent alternative pathogenetic mechanisms both contributing to the ABC-DLBC development by inducing a block in the post-GC B cell differentiation, and these events are also likely to cooperate with the constitutive NF-kB activation (see below) [222, 227].

The SPI-B locus on chromosome 19 (19q13.3-q13.4) is target of both rare chromosomal translocations and genomic gains in ABC-DLBC [161]. SPI-B is an important GC transcription factor regulated by BLIMP1 and significantly more highly expressed in ABC-DLBC than in GCB-DLBC [17]. Accordingly, the silencing of SPI-B expression is
detrimental for ABC-DLBCL cell lines, but it has little or no toxicity for GCB-DLBCL and PMBL [161].

3.2.2 BCR AND NF-KB SIGNALING

NF-κB constitutive activation is the most important alteration found in the ABC-DLBCL subtype also for its possible therapeutic implications [19, 161]. Constitutive activation of NF-κB pathway can occur via genetic lesions of many different genes, targeting the different mechanisms that lead to NF-κB activation in normal GC B cells. Some of these genes are inactivated (TNFAIP3, ITPKB and TRAF3), others are activated (CARD11, CD79B, CD79A, TRAF2, TRAF5, TAK1, RANK, NFKBIZ and MYD88) [206, 207, 228]. TNFAIP3 is one of the most commonly altered, by deletions or mutations, in addition to aberrant SHM, occurring in 20-30% of all DLBCL for the vast majority of the ABC type. The gene codes for a negative regulator of the NF-κB and its lost or inactivation in B cells contributes to the constitutive activation of NF-κB pathways and tumor transformation [19, 229]. In contrary, CARD11 mutations induced constitutive NF-κB activation in the absence of antigen receptor signals (such as CD40-CD40L) conferring a gain-of-function phenotype. The gene coding for MYD88, an adaptor protein that mediates toll-like receptor (TLR) and interleukin-1 (IL1) receptor signaling [161], is recurrently mutated in up to 30% of ABC-DLBCL [228]. MYD88 mutations, mostly represented by a L256P mutation, promote NF-κB activation but also the activation of JAK-STAT3 pathway [230, 231]. Interestingly, MYD88 mutation is often detected together with other mutations: 34% of ABC-DLBCL with MYD88 mutation also carries mutations in CD79A or CD79B genes that act on NF-κB pathway suggesting that these mutations provide non-redundant signals for survival of ABC-DLBCL cells [19].

Gains of chromosome 3, particularly of its long arm, are characteristic of ABC-DLBCL. The fact that the lesions are usual very large suggest that more than one gene might contribute to lymphomagenesis. Among these genes known to be deregulated are BCL6 and FOXP1 [161, 232, 233], mapped at 3q27 and 3p14, respectively, while another candidate gene is NFKBIZ, which encodes an IkB-like protein that binds to NF-κB heterodimers and enhances transactivation of NF-κB targets [192, 207]. NFKBIZ was recently found up-regulated in ABC-DLBCL compared to GCB-DLBCL patients samples also demonstrating that NFKBIZ is essential for nuclear NF-κB activity in ABC-DLBCL [142]. The activation of NF-κB, BCR and JAK-STAT pathways provide therapeutic targets which are currently been explore [234].
3.2.3 CELL CYCLE
Deletion of the INK4/ARF locus on chromosome 9p21 is a lesion more common in ABC-subgroup than in GCB-DLBCL [161]. The INK4/ARF locus encodes three tumor suppressors (CDKN2B, ARF and CDKN2A) and it is among the most frequently inactivated locus in human cancer. CDKN2A together with CDKN2B, CDKN2C and CDKN2D selectively inhibits the cyclin D-associated kinase, leading to a block in G1-S phase. Instead, ARF is a protein that stabilizes TP53 preventing MDM2-mediated proteasome degradation [15]. Cases with del(9p21) show a specific gene expression signature characterized by deregulation of the RB/E2F pathway, activation of cellular metabolism, and decreased immune and inflammatory responses [235]. INK4/ARF locus deletion is associated with a poor outcome in the ABC subtype, defining a subgroup with a particularly unfavorable outcome [161, 235]. Deletion at 9p21 is also an important lesion during biological transformation from an indolent lymphoid tumor to DLBCL. In particular, it is associated with transformation from FL [143, 164] and, from chronic lymphocytic leukemia (Richter Syndrome, RS). In the latter condition, del(9p21) is the most commonly acquired lesion in the aggressive samples [141].

3.2.4 OTHER LESIONS
Similarly, gain of 18q21-q22 occurs more frequently in ABC-DLBCL cases than in GCB-DLBCL and PMBL cases [161]. The two most up-regulated genes in this locus are BCL2 and NFATC1, and both can contribute to the pathogenesis of DLBCL. As already above mentioned, in GCB-DLBCL amplification of the 18q21 locus seems to occur only in cases that have t(14;18), which juxtaposes BCL2 to the IgH locus, never observed in ABC-DLBCL subgroup, and this is associated with high expression of BCL2 gene [209]. Both 3q and 18q gains have been reported to correlate with shorter survival in patients with DLBCL [161, 236], possibly explained by their association with the ABC-DLBCL.

3.3 SHARED LESIONS
3.3.1 IMMUNE SURVAILANCE
Escape from immune surveillance is a necessary step for tumors development [190]. DLBCL down-regulate both major histocompatibility complexes (MHC) class I and II on their cell surface and both genetic and epigenetic mechanisms contribute to this
process [237-241]. B2M (β2-microglobulin) forms the MHC class I with the human leukocyte antigen (HLA) heavy chain. The B2M gene is genetically inactivated in one third of DLBCL, with similar frequency in both GCB and ABC subtypes [192, 242], and a much larger fraction of DLBCL (75%) do not express the protein or have an aberrant pattern of expression [242]. Genetic deletions of the genes coding for MHC class II are common in DLBCL cases arising in immune-privileged anatomical sites such as testis and central nervous system, but less among the remaining cases [238, 240, 241]. Upstream events, namely the down-regulation of the MHC class II transactivator CIITA seems to be another important mechanism contributing to MHC silencing [240, 241, 243]. Genetic disruption of the CIITA gene by chromosomal translocation are common in PMBCL (38% have translocations inactivating the gene) but rare in DLBCL (<5%) [244]. DNA losses are also not common [192, 245], while mutations have been reported but their biologic relevance is unclear [192]. Despite the lack of frequent losses, the loci coding for HLA I and HLA II, both mapped at 6p, are targets of loss of heterozygosity (LOH) [239], namely of copy neutral LOH [186]. The latter phenomenon is less common in DLBCL arising in individuals with an acquired immunodeficiency (ID-DLBCL) such as HIV infection (HIV-DLBCL) or recipients of a solid organ transplant (PT-DLBCL) [246], settings in which the T cell mediated immune surveillance is already reduced. Different other genetic events have been reported to possibly contribute to immune escape.

CD58 is the receptor of the CD2 molecule expressed by T- and NK cells and its expression is necessary for the T- and NK cells mediated cytotoxicity [242]. The CD58 gene has been shown genetically inactivated in 21% of DLBCL, twice more frequently in the ABC (68%) than in GCB (32%), but the protein expression is deregulated in 67% of all DLBCL, with no differences between ABC and GCB types (68% vs 65%) [192, 242]. The above-mentioned rearrangements inactivating the CIITA gene usually also determine the over-expression of the ligands of the receptor molecule programmed cell death 1 (CD274/PDL1 and CD273/PDL2) [243]. Similar rearrangements or DNA amplifications target the two genes also in approximately 20% of DLBCL [192]. Potential therapeutic activity of disrupting the PD-1/PD-L1 pathway is under clinical evaluation in both solid tumors and hematological cancers [128-130, 247]. TNFSF9 gene has been reported inactivated in 12% of DLBCL, possibly affecting the interaction of lymphoma cells with T cell mediated immune surveillance [192].
TNFRSF14, also involved in CD8+ T cells, has been reported to be inactivated in 20% of DLBCL [172]. TNFRSF14 inactivation is observed also in around 20% of FL, in which it is associated with a poor outcome contributing to histologic transformation to DLBCL [248].

FAS (TNFRSF6/CD95) can lead to cell death via the Fas-associated death domain protein (FADD) signaling complex. FAS gene is inactivated by mutations and/or deletions (including homozygous losses) in 5-20% DLBCL cases [171, 249, 250], but the frequency seems higher in cases arising in extra-nodal sites and/or associated with autoimmune disorders [250, 251], in cases belonging to the OxPhos cluster and bearing the BCL2 chromosomal rearrangements.

Similarly to FAS, also genes coding for the TRAIL receptors TNFRSF10A (TRAIL-R1) and TNFRSF10B (TRAIL-R2), are targets of DNA losses in DLBCL [186, 252].

Possible hints regarding a role in immune escape for specific lesions might come from the comparison of lesion associated with immunocompetent DLBCL patients against lesions associated with ID-DLBCL cases. The deletion at 13q14.3, encompassing MIR15A and MIR16 loci, and often also the tumor suppressor gene RB1, is almost never detected in ID-DLBCL [246], indicating that it might contribute to the escape from the immune system control, although the exact mechanism is still unknown, albeit possibly involving FAS [249]. The very low frequency of otherwise commonly observed genomic lesions such as 3q gain in PT-DLBCL [253] or 18q gain in HIV-DLBCL [246] also indicates that genes mapped in these regions might provide specific advantages to skip different types of immune surveillance since the immunodeficiency observed in the HIV or post-transplant settings are different.

An important general notion is that the inactivation of molecules involved in immune surveillance, such as FAS or the TRAIL receptors, does not only provide advantages to protect the lymphoma cells from immune system cells, but also from the apoptotic response following chemotherapy, and this might also explain the reason why some of these defects have been associated with a poor outcome [186].

3.3.2 ABERRANT SOMATIC HYPERMUTATIONS (ASHM)

ASHM result from an aberrant activity of the physiologic SHM process, necessary for the generation of antibodies with high-affinity for antigens [203, 254]. About 70% of DLBCL cases carry ASHM affecting different genes. The mutation rate of an Ig gene (physiologically mutated) is proportional to the transcription rate of the locus, and mutations affect a region of DNA up to 2Kb from the start of the transcription site, which can deregulate the genes or modify the proteins. In a normal GC B cell, SHM process is
not specific for Ig genes. SHMs target also other genes of GC B cells, such as BCL6 and FAS [63, 64, 255] with a frequency 50-100 times lower than the mutations affecting Ig genes and mutations occurring in neoplastic B cells [63]. Mutations in these genes cluster downstream of the promoters as in the case of Ig genes. However, some mutations can occur inside the coding region of genes, such as mutations in exon 9 of FAS detected in some B cell lymphomas [250, 255]. The BCL6 and FAS gene regions do not share primary sequence homologies with the IgV sequences, suggesting that SHM does not require a specific sequence in the promoter of target genes but a kind of specificity regarding the site of mutation at a comparable distance from the transcription initiation site in all these genes [63]. Why and how SHMs can affect non Ig genes in normal B cells is still unclear.

Besides BCL6 others proto-oncogenes are affected by aberrant SHM and they include: MYC, RHOH/TTF, PAX5 [63, 65] IRF4, ST6GAL1, BCL7A, CIITA, LRMP [256], BCL2 [162], and SOCS1 [257]. Aberrant SHM of these genes are found at sites known to be involved in translocations and this suggests that ASHM, requiring double strand breaks, might be involved in the rearrangement.

As a whole, ASHM of PIM1, MYC, RHOH, and PAX5 occurs in about 70% of DLBCL and although are detected in both GCB and ABC-DLBCL, the affected genes seem to differ in the two subtypes: BCL2 and MYC genes are more commonly mutated in GCB DLBCL, while BCL6 in ABC-DLBCL [258], reflecting the different frequency of translocations in the two DLBCL subtypes.

3.4 PMBCL

PMBCL, as said before, is now considered a distinct entity from DLBCL [244] and, for this reason, I will only briefly present it.

The most commonly observed gains are at +2p, +9p, +12q and +Xq whereas common regions of loss occur at 1p, 3p, 13q, 15q and 17p [259]. These patterns of genomic changes are distinct from those observed in GEP-defined DLBCL subgroups. PMBCL shares important oncogenic features with classical HL (cHL) including activation of the NF-kB pathway, but the most important common genomic lesion between PMBCL and cHL is the gain or amplification at 9p24 chromosomal locus the site of the tyrosine kinase JAK2 [15, 16, 19]. This lesion is detected in 45% of PMBCL but less frequent in ABC-DLBCL (11%) and GCB-DLBCL (7%) [161]. Recently, Rui et al. observed 10 genes that were upregulated in PMBCL cases, of which PDCD1LG2, encoding for the T cell inhibitory ligand PD-L2, and JAK2, JMJD2C and RANBP6 [260]. The involvement of the immunoregulatory
Introduction – Genetics of diffuse large B cell lymphoma

genes PD-L1 and PD-L2 were also confirmed by Green et al. using an integrative analysis of high-resolution copy number data and transcriptional profiles [138].

JAK2 encodes for a tyrosine kinase involved in the phosphorylation pathway of STAT transcription factors, which subsequently dimerize and translocate into the nucleus, promoting gene transcription [261]. JAK2 is deregulated also in other cancers [262], and it can represent a therapeutic target. Gene expression studies have documented over-expression of JAK2 in PMBCL and have suggested constitutive activation of the IL-4 and IL-13 pathways as a result [16, 263], including the receptor interleukin-13 receptor alpha-1 (IL-13Rα1) and STATs, such as STAT6, and several downstream target genes, suggesting that this pathway may play an important role in the pathogenesis of these tumors [15].

JMJD2C encodes for a H3K9me3 demethylase and is known to be mutated in other disease. Knockdown of this gene in breast, prostate, and esophageal cancer cell lines suppresses cells proliferation [264, 265].

As above mentioned, frequent gains of 2p14-16 have been documented in PMBCL, implicating the REL proto-oncogene. Despite generally low transcript levels and weak nuclear REL staining in the PMBCL in cell lines MedB-1 and Karpas-1106P [266], these exhibited high NF-kB activity. This might be explained by the short-half of REL mRNA or the activity of regulatory factors such as PU.1 and SPI-B that influence REL transcription.

PMBCL cell line survival is critically dependent upon NF-kB, and the target gene profile of NF-kB in PMBCL is clearly directed towards genes promoting cell survival and inhibiting apoptosis [267]. The constitutive and high NF-kB activity seems to be an important component for PMBCL lymphomagenesis. However, this abnormal state could be caused by different mechanisms with the over-expression of REL as the most likely one.

The CIITA has also been implicated in PMBCL [19, 243]. CIITA is translocated in 40% of PMBCL cases leading to a tumor escape from immune responses.

3.5 UNCLASSIFIABLE CASES OF B CELL LYMPHOMA WITH FEATURE INTERMEDIATE BETWEEN DLBCL AND BL

A borderline category was included in the WHO classification [268] for cases of B cell lymphoma with features intermediate between DLBCL and BL, another aggressive NHL but more curable than DLBCL and derived from GC B cell. Some cases were previously classified as Burkitt-like (BLL) lymphoma also because they are morphologically more similar to BL but had an atypical immunophenotype and genetics features that preclude a
diagnosis of BL. This subtype of DLBCL is characterized by the presence of multiple chromosomal translocations, mostly affecting MYC, BCL2, BCL6 or CCND1 (Cyclin D1). These so called “double” (BCL2 and MYC translocations) and “triple-hit” (BCL2, MYC and BCL6 translocations) lymphomas have a very poor prognosis, both with standard or high-dose chemotherapy, despite usually having a GCB-phenotype [115, 116, 175, 176, 178, 179, 181-183, 269]. As said before, not only the co-occurrence of MYC and BCL2 chromosomal translocations, but also the co-expression of the two proteins determines a very poor outcome. In another study, concurrent expression of MYC positive and BCL2 was observed in 21% of the cases, while MYC translocation only in 11% [185]. Importantly, MYC protein conferred a poor outcome only when co-expressed with BCL2 [185].

### 3.6. REMARKS

DLBCL is the commonest type of non-Hodgkin’s lymphoma (NHL), occurring in approximately 50% of patients. DLBCL is an aggressive disease that remains incurable in about 50% of the patients. The modest therapeutic success is in part due to the remarkable heterogeneity on this disease. DLBCL is divided into various molecular subtypes characterized by different genomic aberrations and outcomes of patients. As shown in this chapter, the dramatic improvement in genomic analysis technologies is providing the means to obtain a comprehensive description of the landscape of genetic lesions that are associated with DLBCL.

Analyzing the whole genome of 166 DLBCL cases, we detected a recurrent DNA gain on chromosome 11q24.3 never described before. Within this gain two genes were expressed in B cells, ETS1 and FLI1. But, is the 11q24.3 important for the prognosis of DLBCL patients? Is also associated with a particular molecular subtype of DLBCL patients? Thus, considering the importance of understanding the observed genomic aberrations, the overall aim of this research project was to functional characterize this lesion affecting ETS1 and FLI1 expression, investigating its role in DLBCL lymphomagenesis.
4. New agents in diffuse large B cell lymphoma patients
A still too large portion of patients with DLBCL, and NHLs in general, succumb to their disease because of primary refractory disease or relapses after first-line therapy.

In DLBCL, the development of the CHOP regimen represent a major milestone. Attempts to improve the outcome of DLBCL patients tried to intensify the therapy by combining additional chemotherapy agents, increasing doses or shortening the interval between the doses. However, these approaches did not bring to sound improvement [270-273] and CHOP (cyclophosphamide, doxorubicine, vincristine and prednisone) remained the standard, although not satisfactory, scheme.

The significative improvement in DLBCL prognosis came with the addition of rituximab (anti-CD20 monoclonal Ab, mAb) to the combination of CHOP (R-CHOP), leading to a dramatic increase in the cure rate for DLBCL patients. However, a significant proportion of patients still experience primary refractory disease or early relapses and poor survival.

Achieving first-line complete remission (CR) is of major importance.

Again, attempts have been made to intensify treatment doses, shortening intervals between cycles or by the use of high dose chemotherapy followed by autologous bone marrow transplantation, but without clear improving the outcome of the patients with aggressive disease [273].

Thus, it is clear the need to introduce new compounds in order to possibly cure these aggressive cases. Increased understanding of the molecular features of aggressive B cell lymphoma has led to the development of a range of novel therapies. Which are now under preclinical and clinical evaluation.

4.1. MONOCLONAL ANTIBODIES (mAbs)

Novel monoclonal antibodies are under evaluation with the aim to improve on the efficacy of rituximab.

Anti-CD20 mAbs have been modified mainly in two manners: 1. By increasing direct cell-death capacities, through modifications of the V regions of the antibody. 2. By increasing immune effector functions such as antibody-dependent cytotoxicity through intrinsic changes of the Fc region of the antibody, the tail region of the antibody that interacts with cell surface receptors.

Veltuzumab is a humanized anti-CD20 monoclonal Ab with complementarity-determining regions differing from rituximab by only one amino acid, leading to an increment in cell cytotoxicity [274]. For this drug, a major response was demonstrated in a phase I/II dose-escalation trial in patients with relapsed or refractory NHL [274]. Like
Vertuzumab, ofatumumab induces B cell depletion via mechanisms similar to rituximab, but with substantially more complement-dependent cytotoxicity, and seems to be more potent than rituximab in both rituximab-sensitive and rituximab-resistant models [275, 276]. Obinutuzumab (GA-101) was designed as a third-generation, humanized, and glycoengeneered anti CD20 antibody [277]. It exhibits a lower complement-dependent cell death but a major cell death induction respect rituximab. In addition, GA101 harbors a glycoengineered, afucosylated Fc segment, that helps in binding affinity for the FcγRIIIa molecule respect rituximab, translating into an increased induction of cytotoxicity. GA-101 development is mainly focused on indolent lymphomas but it has shown activity also in heavily pre-treated patients.

In addition to anti-CD20 mAbs other B cell markers are studied as targets of therapeutic antibodies. Some of these have similar cytotoxic effects of rituximab or its derivative molecules increasing cell death by direct targeting molecules for lymphoma cell survival different to CD20 (e.g. CD79), or to activate target molecules that transmit a death signal (e.g. tumor necrosis factor-related apoptosis inducing ligand receptor: TRAIL-1 or TRAIL-2). Epratuzumab is a humanized mAb that targets CD22, a B cell marker thought to play a role in B cell activation and in modulation of antigen-receptor signaling [278]. Epratuzumab seems to work as first-line therapy for DLBCL when added to R-CHOP in a phase II trial. Lucatumumab (HCD122) is a mAb that is pure antagonist of the CD40 transmembrane receptor and has been evaluated clinically in CLL and multiple myeloma (MM) and is currently under evaluation also in DLBCL. The humanized anti-CD40 mAb, dacetuzumab (SGN-40), has demonstrated antiproliferative and apoptotic activity against a panel of high-grade B cell lymphoma cell lines [279]. Dacetuzumab was shown to enhance the antitumor activity of rituximab in NHL cell lines and xenograft models, suggesting that antibody-mediated signaling through both CD20 and CD40 may be an effective strategy in the treatment of NHL [280].

Another class of rituximab-derived mAbs are bispecific antibodies that simultaneously target CD20 together with other B cell surface markers such as CD22, HLA-DR:INF-αβ, or anti-CD19/CD3 (blimatuminab) that simultaneously target an antigen on malignant cells and CD3 on the surface of T cells.

Finally, newly designed antibody-drug conjugates have shown interesting results. These are mAbs attached to cytotoxic drugs via chemical linkers. Inotuzumab ozogamicin (CMC544) is composed of the anti-CD22 antibody (inotuzumab) links to calicheamicin, a cytotoxic agent derived from the bacteria Micromonospora echinospora, which acts by cleaving DNA [281]. Inotuzumab ozogamicin was well tolerated showing good results.
Introduction - New agents in diffuse large B cell lymphoma patients

during a phase I study with 48 patients with a refractory or relapsed FL or DLBCL [282]. Better results were obtained after combination with rituximab treatment [282]. An open phase I study of inotuzumab ozogamicin is ongoing at IOSI in Bellinzona (NCT01535989).

\(^{90}\)Y-epratuzumab-tetraxetan is a radiolabeled, huminazed anti-CD22 antibody that has been used for radioimmunotherapy and has shown high rates of CR in indolent and aggressive NHL in a phase I/II study [283]. \(^{90}\)Y-ibritumomab tiuxetan \((^{90}\)Y-IT) is another example of mAb used for radioimmunotherapy. \(^{90}\)Y-IT is an anti-CD20 murine antibody linked to a beta-emitting isotope used in addition to rituximab in patients with refractory or relapsed DLBCL [284].

4.2. IMMUNOMODULATORY DRUGS

The tumor microenvironment is a valid therapeutic target because surrounding normal cells provide support for the malignant cell. Lymph node involved by malignant lymphoma contains different type of cells: T cells, dendritic cells, macrophages, and stromal cells. All these play a role in malignant cell growth. Lenalidomide and pomalidomide are immunomodulatory drugs derived from thalidomide, and have already established their role in the treatment of MM and CLL, but recently received increasing interest in NHL [285, 286]. Their mechanism of action is not fully elucidated, but comprises an enhancement of NK cell antitumoral activity and a change in the balance between pro- and anti-inflammatory cytokines (TNF-\(\alpha\), IL2, INF-\(\gamma\), IL6, IL8, IL10 and PDGF factors) leading to increased apoptosis [287]. Moreover, long term treatment of malignant PCs with lenalidomide determines a downregulation of NF-kB activity [288, 289], which results in a reduction of antiapoptotic proteins including cIAP2 [290] and FLIP [291]. Phase I/II studies are reported for these drugs on DLBCL patients [292, 293], and a phase I trial on 20 newly diagnosed DLBCL patients revealed that lenalidomide in combination with R-CHOP resulted in improved responses [294]. Interestingly, recently published data showed a better sensitivity to lenalidomide in non-GCB than in GCB refractory or relapsed DLBCL [295]. A large international trial (NCT01197560) has been opened to enrollment in an attempt to prospectively validate these retrospective observations.
4.3. CYTOTOXIC AGENTS

Several new cytotoxic agents are being investigated for the treatment of aggressive lymphomas. Since cancer cells, in general, proliferate faster than healthy cells, cancer cells are more sensitive to DNA damage such as alkylation. Alkylating agents are used to treat several cancers. However, they are also toxic to normal cells, leading to damage, in particular in high proliferating cells, as those in the gastrointestinal tract, bone marrow, testicles and ovaries. Bendamustine has a chemical structure that suggests the possibility of both alkylator-like activity as well as that of purine nucleosides. It has shown single-agent and combination activity in indolent lymphomas. Study of bendamustine in combination with rituximab in relapsed or refractory aggressive B cell NHL confirmed that it was feasible and well tolerated and showed promising efficacy [296, 297]. Also combination of gemcitabine and oxaliplatin (GEMOX) is effective in relapsed DLBCL and well tolerated by patients not fit enough to undergo high-dose chemotherapy and stem cell transplantation. Phase II studies have demonstrated significant activity of GEMOX in combination with rituximab (GEMOX-R) in relapsed DLBCL [298].

Another new cytotoxic drug is pixantrone dimaleate an aza-anthracenedione. Pixantrone was proposed as a therapy for NHL patients who had progressed or relapsed after anthracycline-containing treatment regimens because it was well tolerated by these aggressive NHL patients. A phase III study was conducted to evaluate pixantrone as a single agent compared to the investigators’ choice of single-agent chemotherapies in NHL patients who had relapsed after two or more prior chemotherapy regimens. From this study pixantrone-treated patients had a higher CR rate compared with the control group, but a higher frequency of cardiac adverse events was observed, may due to randomization effects (five patients in the pixantrone group had histories of congestive heart failure or cardiomyopathy) [299].

4.4. INHIBITORS OF SIGNALING PATHWAYS

Multiple signaling pathways appear to play a significant role in lymphoma growth and survival as they cause the constitutive activation of pathways such as NF-kB, Jak/Stat, RAS and MAPK pathways. Inhibiting these pathways might result in significant clinical benefit for patients.

Fostamatimib disodium (R788) is a tyrosine kinase inhibitor targeting spleen tyrosine kinase (SYK). SYK is known to play a role in immune receptor signaling because is associated
with the cytoplasmic domain of BCR leading to survival and proliferation intracellular signals. There are evidences that SYK is required during lymphomagenesis [300, 301]. Fostamatimib disodium has been shown to decrease BCR signaling via SYK inhibition and can lead to apoptosis in DLBCL cell lines [302]. In clinical studies this compound has shown promise in a phase I/II trial [303]. As BCR is constitutive activated in ABC-DLBCL subtypes of patients, may be therapeutic strategies involving BCR signaling inhibitors affecting the BCR and the NF-kB pathways may be used to treat ABC-DLBCL with more efficacy [304].

Another promising approach is the inhibition of bruton tyrosine kinase (Btk). In a normal B cell context Btk activation triggers a cascade of signaling events that culminates in the generation of calcium mobilization and fluxes, cytoskeletal rearrangements and transcriptional regulation involving NF-kB. Ibrutinib (PCI-32765) directly inhibits Btk activity preventing B cell activation as well as the growth of malignant B cells. Phase II trials were performed on different NHL [305] patients. In DLBCL cell lines the cytotoxic effects of combination of ibrutinib and lenalidomide have been observed especially in ABC-DLBCL bearing mutations of MYD88 but no toxicity was observed in GCB-DLBCL lines that lack oncogenic activation of the BCR and MYD88 pathways [289]. Cytotoxic effects are due to the synergy between ibrutinib and lenalidomide in blocking IRF4 expression and in increasing IFNβ production, which is toxic for ABC-DLBCL cell lines [289]. A phase III study of ibrutinib in combination with R-CHOP is now running (NCT01855750).

4.5. PI3K/AKT/mTOR PATHWAYS INHIBITORS

The PI3K/AKT/mTOR pathway is an intracellular signaling that plays a key role in cell metabolism, proliferation, and survival and it is often deregulated in cancer. In solid tumors, the deregulation of PI3K/AKT/mTOR pathway is the result of mutation of PI3K, or amplification of AKT. AKT is downstream of PI3K and upstream from mTOR. mTOR activation by AKT leads to cell proliferation and survival by modulating protein synthesis of critical molecules such as cyclin D1. mTOR signaling also activates NF-kB. In many cancers, PI3K/AKT/mTOR pathway is overactive, thus reducing apoptosis and allowing proliferation. Consequently, some experimental cancer drugs aim to inhibit the signaling sequence at some point. Direct inhibition of PI3K can potentially lead to inhibition of AKT and mTOR.

Idelalisib, GS-1101 (CAL-101), is a selective and potent inhibitor of the PI3K isoform p110δ, which is predominant in hematologic cells, and has entered phase I testing were it shown
acceptable safety and promising pharmacodynamic and clinical activity in a variety of hematologic malignancies, as single agent as in combination with rituximab or bendamustine [306].

Perifosine is a novel oral agent in a new class of cancer therapies, the alkylphospholipids. Within its drug effector function it blocks activation of AKT leading to cell death. However, perifosine may also acts by other means, including effects on the MAPK and JNK pathways [307]. Perifosine has been studied in gastrointestinal, renal cancers, and hematopoietic malignancies, including MM and Waldenström macroglobulinemia. A phase II trial of single agent was performed in patients with released or refractory Waldenström macroglobulinemia [308]. Temsirolimus (CC-779) [309], everolimus (RAD001) [310], and deforolimus are some of the mTOR inhibitors under clinical evaluation, alone or in combination. mTOR is represented by 2 components, mTORC1 and mTORC2. Only the mTORC1 component is inhibited by rapamycin and the rapalogs. Clinical trials are currently underway to test the efficacy of these compounds. Temsirolimus was mainly studied in mantle cell lymphoma (MCL), but it also shows some activity in DLBCL [311]. Everolimus has shown activity in a variety of hematologic neoplasms. This oral agent was tested in pre-treated aggressive lymphoma cases [312]. The overall response rate was 32% with a median duration of response of 5.5 months. These data confirmed that mTOR inhibitors have significant activity in malignant lymphoma, giving proof to the concept that targeting mTOR is relevant in this disease. SF1126 is a dual PI3K/mTOR inhibitor and is currently in phase I development in B cell malignancies [313].

4.6. KINASE INHIBITORS

I already mentioned some kinase inhibitors (SYK and Btk inhibitors), but different others compounds are currently tested targeting different kinase proteins.

Aurora kinases A is a mitotic regulating serine/threonine kinase that is over-expressed in a number of malignancies. Over-expression of Aurora kinases during the cell cycle can override mitotic and spindle check-points leading to aneuploidy in many human cancers. Alisertib (MLN8237) is a Aurora kinases A inhibitor, which in a phase II study in patients with multiple relapsed and highly refractory aggressive B cell and T cell lymphomas, show durable responses. Alisertib is also being studied in combination with rituximab, with or without vincristine, in relapsed and refractory DBLCL or transformed FL. Another kinase, target of new drugs, is the protein kinase C (PKC), of which over-expression have been associated with a less favorable outcome in DLBCL [314].
Enzastaurin is an inhibitor of PKC-β studied in a phase II study with relapsed and refractory DLBCL patients. From this study prolonged freedom from progression (FFP) was observed with little grade 3 toxicity [315]. Preliminary results from a subsequent study in aggressive NHL also indicate single-agent activity [316]. A phase III study with daily enzastaurin to prevent relapse in DLBCL patients in remission after R-CHOP treatment is currently ongoing (NCT00332202).

4.7. PROTEASOME INHIBITORS

The proteasome is a multicatalytic proteinase complex responsible for the degradation of misfolded or unneeded proteins by proteolysis. Most of these intracellular proteins are crucial to cell cycle regulation, programmed cell death, or apoptosis. At first, these proteins are tagged for degradation with a small protein called ubiquitin. The tagging reaction is catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein. As the essential role of the proteasome in cell function, transformed cells display greater susceptibility to proteasome inhibition than nonmalignant cells. Therefore, proteasome inhibition holds promise as a novel approach to the treatment of cancer. After proteasome inhibition, several important proteins are accumulated inside the cell, such as IκB, the inhibitor of NF-κB, the tumor suppressor p53, the cyclin-dependent kinase inhibitors p21 and p27, and the proapoptotic protein Bax. Accumulation of these substrates leads to inhibition of NF-κB crucial to the promotion of tumorigenesis. Thus, the first proteasome inhibitor, bortezomib, has become an important treatment in the management of multiple myeloma and has more recently been shown to have activity in lymphoma. Interim data from a phase II study suggested promising results for a regimen of bortezomib plus CHOP every 2 weeks as first-line treatment in DLBCL [317]. Another study showed that although bortezomib alone had no activity in DLBCL, when combined with chemotherapy it demonstrated a significantly higher response in ABC- compared with GCB-DLBCL [318].
4.8. EPIGENETIC INHIBITORS

Proper chromatin regulation is fundamental in controlling gene expression and critical for cellular processes, including self-renewal, differentiation and proliferation [319-322]. The epigenome is in a highly dynamic condition due to precise temporal and spatial chromatin modifications. Three classes of proteins involved in chromatin regulation can be recognized: epigenetic “writers”, “erasers” and “readers”. “Writers” add chemical modifications to DNA or histones (for example, DNA methyltransferase). “Erasers” remove these modifications (for example, HDAC). “Readers” start regulatory actions, such as initiating/silencing transcription or DNA repair, based on the pattern of chromatin modifications. Importantly, chromatin modifications can be manipulated and reversed, providing the rational to pharmacologically target the epigenome. Considering the frequent deregulation of genes coding for chromatin regulators in DLBCL, targeting of epigenetic factors is a rational therapeutic strategy.

Several groups of HDAC inhibitors (HDACi) have been developed such as vorinostat (SAHA, Zolinza), approved for treatment of cutaneous lymphomas (CTCL). Vorinostat role in the treatment of DLBCL is not clear yet. A number of phase I studies of vorinostat-combination regimens are either ongoing or have been recently completed. These studies have incorporated rituximab [323] or pegylated liposomal doxorubicin, and preclinical evidence supporting the clinical development of vorinostat plus the novel Aurora kinase inhibitor, MK-5108 [324]. Panobinostat is another HDACi that has shown activity in a variety of cancers. Responses have been documented in a phase II study in relapsed HL [325]. It is also being investigated in DLBCL, where preclinical activity has been observed in combination with the cytidine azanucleoside analog, decitabine, a drug which has direct cytostatic activity [326].

Methylation of histones H3K9 and H3K27 is generally associated with transcriptional repression and often concurrent with deacetylation. EZH2 is the catalytic component of the polycomb repressive complex 2 (PRC2) responsible for the methylation of H3K27 and, subsequently, for the repression of select genes. EZH2 mutations on residues Y641 and A677, within the catalytic domain of the protein, were identified in 22% of DLBCL and 10% of FL patient samples, resulting in alteration of EZH2 activity, in particular, in the enhancement of di- and trimethylation of H3K27. McCabe at al. [200] demonstrated that the S-adenosylmethionine (SAM)-competitive small-molecular inhibitor GSK126 selectively inhibits EZH2 methyltransferase activity in GCB–DLBCL, decreases global H3K27me3 levels and reactives silenced PRC2 target genes. Interestingly, GSK126 inhibits the proliferation of EZH2 mutant DLBCL cell lines and the growth of EZH2 mutant DLBCL
Introduction—New agents in diffuse large B cell lymphoma patients

xenografts in mice [200]. These pre-clinical data suggest that pharmacological inhibition of EZH2 activity might be a promising treatment for EZH2 mutant lymphoma.

BET family of bromodomain-containing proteins have also a pivotal role regulating the transcription of cell cycle regulators and growth-promoting genes such as MYC. The BET family includes four members (BRD2, BRD3, BRD4 and the testis-specific isoform BRDT), with each containing two N-terminal bromodomains (BRDs) which recognize epigenetic chromatin modifications, such as histone acetylation at lysine residues. Interestingly, in contrast to other BRD-containing proteins and transcription factors, BET proteins remain associated with condensed and hypoacetylated chromatin suggesting a role in epigenetic memory [327, 328]. Recently, a new therapeutic approach consisting in the use of BET-bromodomain chemical inhibitor as has been studied for MYC-dependent malignancies. In fact, the BET domain containing transcriptional regulator BRD4 was shown to bind to the MYC promoter region playing a critical role in the activation MYC expression [117-119]. I have also contributed to study one of the BET bromodomain inhibitor, OTX015, in DLBCL.

4.9. BCL2 INHIBITORS

The Bcl- family proteins (BCL2, BCL-XL, BCL-W, etc) are key regulators of cell survival through their effects on the mitochondrial-mediated pathway of apoptosis, or programmed cell death. This family of proteins includes proapoptotic and antiapoptotic proteins, and the balance of these can control whether a cell lives or dies and cancer cells are known to have altered expression of these proteins. Thus, targeting Bcl- family proteins has therefore been a goal for the treatment of cancer. The BCL2 antisense nucleotide, oblimersen, showed promising results in a phase II study in combination with rituximab in patients with recurrent B cell NHL [329]. ABT-263 (navitoclax) has been investigated in clinical trials of lymphoma, as monotherapy or in combination with rituximab [330]. This drug inhibits multiple Bcl- family proteins. ABT-263 and its structural analogous ABT-737 are in preclinical development also for DLBCL. However, its development might be hampered by systemic toxicity due to the inhibition of BCL-XL, an important factor for platelets survival. Other agents in preclinical development include obatoclax (in combination with bortezomib) with relapsed MCL and others NHLs [331]. ABT-199 is an orally bioavailable, second-generation BH3-mimetic that inhibits BCL-2 with less activity against BCL-XL. ABT-199 demonstrated single agent anti-tumor activity in
patients with NHL, including DLBCL and MCL [332]. Promising results was also observed for refractory CLL patients [333].

4.10. IMMUNE CHECKPOINTS INHIBITORS

Immune checkpoints refer to inhibitory intracellular pathways, starting from surface antigens or receptors (immune-checkpoint proteins), which allow cells to maintain self-tolerance and to modulate the duration and the amplitude of physiological immune response in order to minimize collateral tissue damage in peripheral tissues.

In adaptive immune resistance, the tumor uses immune-checkpoint proteins in order to protect itself from an antitumour immune response.

T cells are the more interesting cells for therapeutic manipulation because of their several functions in the immune system. As a general rule, in cancers, surface receptor controlling T cell activation are not necessarily over-expressed than in normal cells, whereas inhibitory ligands and receptors that regulate T cell effector functions in tissues are commonly over-expressed on tumor cells or on non-transformed cells in the tumor microenvironment. Thus, these last T cell surface molecules are the most druggable.

Recently, antibodies targeting immune checkpoint proteins on T cells begin to be studied. These types of drug therapies are not designed directly against tumor cells, instead they target lymphocyte immune checkpoint proteins in order to enhance endogenous antitumor activity. The two immune-checkpoint receptors that have been most studied are cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1), both expressed by T cells.

CTLA4 is expressed exclusively on T cells, of which cytotoxic CD8+ T cells and Tregs, where it counteracts the activity of CD28, a T cell co-stimulatory receptor, with which CTLA4 share identical ligands: CD80 and CD86. Once antigen recognition occurs, CD28 signaling strongly amplifies TCR signaling to activate T cells and it has been proposed that CTLA4 expression reduce the activation of T cells competing with CD28 for CD80 and CD86 binding. Ipilimumab and trelimumab are two humanized antibodies used in clinical trials as single agents or in combination [334, 335]. Better results were observed for patients treated with anti-CTLA4 antibodies than patients treated with vaccines alone [336]. A phase III clinical trial was completed with tremelimumab in patients with advance melanoma but the trial showed no survival benefit [337]. The major problem of targeting these molecules is the immune-related toxicity (high grade of inflammation or autoimmunity origin) involving various tissues.
Conversely, the principal role of PD1 is to limit the activity of T cells in peripheral tissues during inflammatory response once T cell became activated. PD1 is expressed also on the surface of other immune cells such as NK cell and B cells.

Increased PD1 expression on CD8+ T cell may either reflect an anergic or exhausted state against the tumor. PD1 has two specific ligands, PDL1 and PDL2. Up-regulation of PD-L1 occurs on a wide variety of human tumors suggesting that cancer cells co-opt the PD-1/PD-L1 inhibitory pathway to evade the host immune response [338, 339]. PDL2 is highly up-regulated on cells from certain B cell lymphomas, such as PMBCL, FL and HL [340], and its up-regulation is associated with amplification or rearrangement of CIITA gene.[243]

Different drugs were designed against PD1 or its ligands. In the first phase I clinical trial with a fully human IgG4 PD1 antibody (MDX-1106), there were some cases of solid tumor regression, including mixed responses, partial responses and a complete response [341]. An advantage of anti-PD1 treatments is the less immune-related toxicity than anti-CTLA4 treatments because of PD1 is expressed only on peripheral immune cells. A phase II trial using pidilizumab, an anti-PD1 antibody, on DLBCL patients after allogeneic hematopoietic stem cell transplantation (AHSCT) demonstrated good results. Thus, pidilizumab may represent a promising therapeutic strategy also in the cure of DLBCL [342].

4.11. REMARKS

Despite the high number of drugs developed against DLBCL lymphoma, a still too large fraction of patients is still not cured. Moreover, some drugs described in this chapter, such as cytotoxic agents, target cellular mechanisms that remain of high importance also in the normal cells, causing toxicity in patients. Recently, there has been a shift from using combinations of cytotoxic agents to selecting molecules that target specific pathways involved in signal transduction, apoptosis, and differentiation and that show specificity for each DLBCL subtypes. Antibodies targeting immune checkpoint proteins that stimulate and not suppress the immune system response also represent a very promising approach.

The new results reported in this thesis, with the identification of ETS1 and FLI1 as two new oncogenes involved in DLBCL lymphomagenesis, represent an original discovery that could have a role in the treatment of DLBCL patients. Thus, I also evaluated the possibility of using ETS1 and FLI1 as therapeutic targets for DLBCL patients.
Purposes
PRIMARY AIM:

Diffuse large B cell Lymphoma represents the most common form of B cell NHL characterized by an aggressive course and developed from GC B cells. It is a heterogeneous group of disorders having numerous alterations and comprise at least two main distinct subtypes characterized by having specific gene expression profiles as derived from B cells at different stages of maturation towards PCs: germinal center B cell-like DLBCL (GCB) and activated B cell-like DLBCL (ABC). GCB-DLBCLs are derived from GC B cells and, accordingly, express genes that are detectable in normal GC B cells. In contrast, ABC-DLBCLs originate from activated B cells that are differentiating into PCs. Both DLBCL subgroups are addicted to different oncogenic pathways due to divergent genetic aberrations. Regarding that point, a hallmark of ABC-DLBCL cases is their dependency to constitutive activity of the oncogenic NF-κB pathway that is caused by recurrent mutations of its regulators. In contrast, GCB-DLBCL are characterized by different genetic aberrations, such as BCL2 translocations leading to inhibition of apoptosis, or by somatically acquired mutations affecting EZH2 that affect chromatin remodeling process.

Thus, normal GC B cell differentiation requires a complex transcriptional program and alterations of genes involved in this process are relevant for DLBCL pathogenesis. Identification and functional characterization of new genetic lesions would help in reduced difficulty in understanding the pathogenesis of DLBCL, and finally, leading to the design of better therapies for the patient.

Starting from this concept, as first aim of this project, I characterized a new recurrent lesion at chromosome locus 11q24.3 that our group had previously found in DLBCL patients. The lesion was a gain and integration of genomic profile with gene expression analysis reveals two genes, ETS1 and FLI1 to be expressed at higher levels in patients carrying the 11q24.3 gain respects the patients without the gain.

ETS1 and FLI1 were already described as important factors during early GC B cell developmental phases regulated by Ca^{2+}-loaded Calmodulin (CaM) pathway, but their role is not so clear yet (Fig.12). In contrast, both had not been described as involved in DLBCL pathogenesis before, and the finding of 11q24.3 gain suggested a potential role in lymphomagenesis for the two ETS factors.

In this thesis, I will try to answer the following questions in order to achieve the first aim: what is the meaning of the 11q24.3 gain? Are ETS1 and FLI1 essential for DLBCL
Purpose

lymphomagenesis in patients with 11q24.3 gain? How ETS1 and FLI1 can be involved in DLBCL lymphomagenesis? Thus, the second and main aim of this project was to functionally characterize the role of ETS1 and FLI1 in DLBCL cases bearing the 11q24.3 gain and, then, to understand if their role could be expanded to DLBCL in general.

Figure 12: Regulation of ETS1 and FLI1 in a normal B cell context. ETS1 and FLI1 are expressed during early GC phases (left part) and must be down-regulated to allow the entrance into PC differentiation, as happen for others important GC factors (BCL6, PAX5, SPI-B) after NF-kB activation. BCR stimulation by antigens exposed by FDC is one of the known mechanisms that initiated PC differentiation. Ca2+ signaling is triggered through BCR followed by E2A down-regulation and the subsequent ETS1 and FLI1 down-regulation. Other mechanisms could be possible but have not been reported yet. Black lines with stop bars indicate inhibitory regulations. Green and black arrows indicate activating regulations of specific genes, pathways or progression in B cell differentiation stages. Blue arrow indicates down-regulation of the alongside gene. Red arrows indicate up-regulation of alongside genes.
SECONDARY AIM:

During the introduction has emerged the importance of genomic lesions for the prognosis of patients for the kind of regime to follow. One of the most study gene involved in DLBCL is MYC because of its oncogenic activity and high deregulation frequency. Different studies demonstrated that MYC translocations are significantly associated with worse survival in patients with DLBCL treated with R-CHOP [115, 116, 120, 121, 180, 184]. Whilst the clinical significance of the presence of MYC rearrangements is established, the clinical implications of extra copies of the gene are less clear. A group of research examined the significance of increased MYC gene copy number in a small series of 30 DLBCL cases, of which only 13 were treated with R-CHOP [176]. No statistical differences in outcome were observed, but the eight cases with MYC gains had a 2-year OS of 43% compared to 73% in the 22 cases without increased MYC copy number. Previously, both the presence of MYC translocations and gains have been associated with a poorer outcome in a series of 156 patients with DLBCL treated with CHOP [187].

With the aim to assess the role of extra copy number of MYC gene in the prognosis of DLBCL patients treated with R-CHOP, the association between the presence of MYC gain and the overall survival of DLBCL patients was analyzed.
Materials and methods
1. PREVIOUS ANALYSIS

TUMOR PANEL

DNA profiles, obtained using the GeneChip Human Mapping 250K NspI (Affymetrix, Santa Clara, CA), of 166 DLBCL samples from a previously published series were investigated (GSE15127) [186]. Genomic profiles of DLBCL cell lines were obtained using the 250K or SNP 6.0 array, as previously described [186].

2. CLINICAL SAMPLES

GENE EXPRESSION AND DATA ANALYSIS

Gene expression data of DLBCL clinical specimens were obtained using the Affymetrix Genechip U133 plus 2.0 (Affymetrix) [GSE10846]. CEL files were imported and normalized using robust multi-array average (RMA) algorithm in Partek Genomics Suite 6.4 (Partek, St. Louis, MO). Box-plots were created with Stata/SE v.12.1 (StataCorp, College Station, TX), and differences of expression between cases with and without the 11q24.3 gain were evaluated with a 1-sided t test for unpaired data with unequal variance. The distinction of DLBCL cases in GCB or in ABC was available in 134 cases: 77 cases by immunohistochemistry according to the algorithm of Hans et al [33] (28 GCB, 47 non-GCB) and 57 by GEP [161] (30 GCB, 27 non-GCB/ABC), as previously described [186]. All patients provided informed consent in accordance with the hospital’s institutional review board and the Declaration of Helsinki.

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded 4-μm-thick sections of DLBCL cases (n=12) and normal tonsil from the San Raffaele Hospital underwent immunohistochemical characterization. Antibodies used were anti-ETS1 (1G11, Novocastra, Nunningen, Switzerland) and anti-FLI1 (G146-222, BD Pharmingen, Buccinasco, Italy).
Material and methods

3. HUMAN CELL CULTURE

CELL LINES
DLBCL cell lines (OCI-LY7, SUDHL-4, Val, U2932, SUDHL-2, and OCI-LY10) were obtained from Dalla-Favera and Staudt laboratories. OCI-LY7, SUDHL-4, VAL, U2932, PC3 and DU-145 were maintained in RPMI1640 supplemented with 10% FCS and 1% penicillin/streptomycin and 2 mM glutamine. OCI-LY7 medium was additioned with nonessential amino acids (100 mM), Na-pyruvate (1 nM), and β-mercaptoetanol (50 mM). SUDHL-2 and OCI-LY10 were maintained in Iscove modified Dulbecco medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM glutamine. MCF7 and HEK-293T cells were cultured under standard conditions (37°C in humidified atmosphere, with 5% CO2) in Dulbecco’s Modified Eagle medium supplemented with 10% fetal calf serum (FCS).
All the cell lines have undergone karyotypes analysis (conventional or with the addition of FISH probes when needed, done by our collaborator M.G. Tibiletti) and search for Mycoplasma infection by biochemical assay (Lonza, San Diego, CA, USA). Controls have been repeated during the course of the project.

shRNAs AND PLASMID CONSTRUCTIONS

The shRNAs were obtained from the Expression Arrest TRC library (Sigma-Aldrich, St. Louis, MO). Human pLKO.1 lentiviral shETS1 used were TRCN0000005588 (sh60A) and TRCN0000005591 (sh60D), shFLI1: TRCN0000005324 (sh61C) and TRCN0000005326 (sh61E). pLKO.1 GFP expressing vector was constructed as previously described [124], pWPI and pCDH expressing vectors were kindly provided by Laura Pasqualucci (Irving Cancer Research Center, NY) and Silvia Monticelli (IRB, Bellinzona) respectively. ETS1 expressing vector was cloned in pCMV6-Entry vector (Origene, Rockville, MD, USA).

TRANSIENT TRANSFECTION WITH LIPOFECTAMINE.

Transient transfection was performed with Lipofectamine (Invitrogen Life Technologies, Carlsband, CA, USA). Lipofectamine is a common trasfection reagent used to introduce siRNA or plasmid DNA into in vitro cell cultures by lipofection. Lipofectamine reagent contains lipid subunits that can form liposomes in an aqueous environment, which capture the transfection materials, such as DNA plasmids. The DNA-containing liposomes can fuse with the plasma membrane of living cells, create pores along the membrane allowing nucleic acids to cross into the cytoplasm and contents to be available to the cells.
cell for replication or expression. For each condition, 0.3 × 10^6 cells were seeded in a 24-wells plate in 750 ml of Optimem medium (GIBCO) and transfected with 250ml of transfection mix containing 50nM or 70nM of siRNA against each genes and the scramble control with the same Lipofectamine rate. After 6 hours the medium was replaced with 500 µl of OCI-LY7 growth medium and cells were incubated for 48, 72 or 96 hours. A second transfection was performed with the same condition after 48h. The cells were incubated for a total of 96h in a 5% CO₂ atmosphere at 37°C.

**TRANSIENT TRANSFECTION WITH JET PRIME**

JetPrime (Polyplus Transfection, Illkirch, France) reagent was used to transfect HEK-293T cells. JetPRIME polymer is a nonlipid transfection reagents that is able to interact with the nucleic acid cargo and the cellular membrane thanks to its positive charge. The complex JetPrime-DNA enters into the cell via endocytosis. However, JetPrime buffering leads to osmotic rupture of the endosome followed by the released of the DNA into the cytoplasm. This mechanism is known as proton sponge-mediated endosome escape. According to the manufacturer’s instructions, 5 × 10^6 or 6 × 10^6 HEK-293T were seeded in T75 cm² flasks. 500µl of reaction mix were prepared per condition containing three different plasmids (third-generation helper plasmids): 3.75 µg of pCMV-dR8.74 packaging vector, 1.25 µg of pMD2.VSVG enveloped vector and 5µg of pLKO.1 or PWPI lentiviral transfer vectors. 20 µl of JetPrime reagent per condition were added to the mix. The mix was incubated at room temperature (RT) for 10’ and then added to each flasks containing cells and mixed gently by rocking the flask back and forth. Cells were incubated for 4 hours at 37°C in a CO₂ incubator and medium changed.

**ELECTROPORATION WITH BIO-RAD Gene Pulser II**

1.5 x 10^5 cells of OCI-LY7 cell line were suspended in 350µl of Optimem medium (GIBCO) and electroporated in sterilized cuvette using 200nM of SiRNA for ETS1, FLI1 and scramble control. Electroporation was performed using BIO-RAD Gene Pulser II with 975 µF and range of voltages from 250 to 400 V; then cells were transferred in a 24 multiwells plate and 650 µl of RPMI 1640 growth medium were added to each sample. The electroporated cells were incubated in a 5% CO₂ atmosphere at 37°C for 48h.
ELECTROPORATION WITH AMAXA OPTIMIZATION NUCLEOFECTOR

Transient transfection of OCI-LY7 cell line was performed using Amaxa Optimization Nucleofector method (Amaxa Inc., Gaithersburg, MD, USA). For each condition, 4x10^6 cells were resuspended in 82 μl of Nucleofector solution V (Nucleofector kit) together with 18 μl of supplements. 2.5 μg of pCMV6-ETS1 or control pCMV6-EV plasmid DNA were added to each cell suspensions and transfered to an Amaxa-certified cuvette. Transfection with each plasmid was carried out using O-17 electroporation program. Immediately after electroporation, 500 μl of prewarmed specific OCI-LY7 medium was added to the cuvette and cells were transferred into culture plates containing 4 ml of prewarmed medium.

LENTIVIRAL INFECTION

Lentivirus supernatant from each HEK-293T transfected cells was harvested after 48 hours of incubation at 37°C in a CO₂ incubator, filtrated (0.22-μm pore), and used directly, to infect exponentially growing cells (1 ml of supernatant every 3 x 10^5 cells). The infection was performed using 24-well plates and a dose of 6 μg/ml of polybrene was added to each well, to allow the interaction between virus and target cells. One cycle of spin infection was performed and cells were left for 5 hours at 37°C, 5% CO₂ to allow the interaction between virus and target cells. Then the cells were washed with PBS 1X and re-suspended in fresh complete medium or in lentiviral supernatant for the second infection and incubated over night. 48 hours after infection puromycin was added to the medium. Clones were selected for 72 hours with the antibiotic. Cells were collected at different days after infection, RNA and proteins extracted and then processed respectively for quantitative real-time PCR (qRT-PCR) and western blotting (WB) analysis.

4. PHENOTYPIC ASSAYS

GROWTH CURVE

Infected cells were recovered and an equal number of cells were plated in triplicates in 24-well plate after puromycin selection. Cells were collected, diluted 1:1 with Trypan Blue and counted daily up to 5 days after the recovery.
**EdU ASSAY**

For EdU labeling Click-iT EdU Flow Cytometry Assay Kits (Invitrogen Life Technologies, Carlsband, CA, USA) was used. Very briefly, 10^6 OCI-LY7 cells were pulsed with EdU (10 mM) for 2 hours at 37°C in 5% of CO. Cells were harvested and fix for 15’ at RT, protected from light. After fixation cells were washed with a saponin-based permeabilization and wash reagent and resuspended in 500 μl of the reaction cocktail containing PBS, CuSO4, Alexa Fluor 488 azide and a reaction buffer additive. Cells were incubated for 30’ at RT and washed like before. Cells were resuspended in 500 μl of PBS with 1% BSA and 2 μl of DNA stain 7-AAD were added to cells. Through BD-FACSCalibur flow cytometer (Becton Dickinson, USA) the covalent reaction between an alkyne of EdU molecule and an azide coupled to Alexa Flour 488 dye, was detected. A copper molecule that is added during the analysis catalyzes this reaction. EdU incorporation was analyzed using the CellQuest Pro (Becton Dickinson).

**COMPETITION ASSAY**

For competition assay OCI-LY7 cells infected with pLKO.1 shGFP, shETS1, or shFLI1 (GFP negative) were mixed with an equal number of OCI-LY7 cells infected with pLKO.1 GFP-expressing vector (GFP positive) in the appropriated medium and the percentage of GFP positive cells was evaluated by fluorescence-activated cell sorter analysis at days 3, 7, 10, 17, and 24.

**APOPTOSIS ASSAY**

Cells previously infected were harvested and washed once in PBS 1X and then stained with 5 μl of Annexin-V-FITC (eBioscience). Briefly, Annexins are a family of calcium-dependent phospholipid-binding proteins that preferentially bind phosphatidylserine (PS). Under normal physiologic conditions, PS is predominantly located in the inner side of the plasma membrane. Upon initiation of apoptosis, PS is translocated to the extracellular membrane as a marker for phagocytosis and is detected by AnnexinV labeled to FITC fluorochrome. The association of AnnexinV-FITC with propidium iodide (PI) allows discriminating early apoptosis to late apoptosis. In fact, PI enters inside the early apoptotic cells through the membrane and intercalates to the DNA, while, in late stage apoptosis, the cell membrane loses integrity thereby allowing AnnexinV to also access PS in the interior of the cell. Cells stained with Annexin-V-FITC were incubated at RT without light for 10’. After
two washes in PBS1X, 10 μl of PI (Sigma) were added in 200 μl of Binding buffer 1X. Cell death was analyzed using a BD-FACSCalibur flow cytometer (Becton Dickinson, USA). The analysis of the percentage of cell death was performed using the CellQuest Pro (Becton Dickinson).

**CELLULAR PROLIFERATION ASSAY (MTT ASSAY)**

Cells were grown at plating density of 20,000 cell/well in a 96-well plate. Small molecule or vehicle alone (DMSO) were added to cells in appropriate growth media the day after plating. After 72 hours, OD of viable cells were quantified by spectrophotometer after 4 hours of 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenil-3H-tetrazolium bromide (MTT) treatment. IC50 values were calculated by sigmoidal dose-response curve fit using BioDataFit program.

**5. MOLECULAR ANALYSIS**

**RNA EXTRACTION AND RETRO-TRANSCRIPTION**

Total RNA was extracted using the TRIZOL reagent (Invitrogen Life Technologies, Carlsband, CA, USA) and purified using the RNA easy kit (Qiagen AG, Hombrechtikon, Switzerland). The concentration of total RNA was determined at 260 nm using the NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA). 1μg of total RNA was reverse-transcribed using the Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Karlsruhe, Germany). Briefly, 1μg of total RNA was combined to 10 μl of 2X RT Reaction Mix and 2 μl RT Enzyme Mix and the final volume of 20 μl was reached with DEPC water. The mix was incubated at 25°C for 10’, at 50°C for 30’ and at 85°C for 5’ in a Tetrad 2 Peltier Thermal Cycler (BIO-RAD). The reaction was then chilled on ice for at least 1’ and 1 μl of RNase-H was added in each tube, with subsequent incubation at 37°C for 20’. The results were diluted 1:5 with DEPC water.
Material and methods

**PCR AND QUANTITATIVE REAL-TIME PCR (qRT-PCR)**

cDNA solution (2.5 μl) was amplified by PCR in a 25 μl solution containing 20 nM Tris-HCl, 50nM KCl, 1.5 nM MgCl$_2$, 5nM dNTPs, 2.5 U Taq DNA polymerase (Roche) and 30 pmol of each primer. The oligonucleotides used for PCR were designed for FLI1 (primer F 5'-GTGCACAGGGGAGTAGG-3'; primer R 5'-TCACTGGCTGATGATCCAC-3'), ETS1 (primer F 5'-GGAGCAGCCGCTCATCTTC-3'; primer R 5'-TTGGAATCCAGCAAGCAT-3'), and GAPDH (primer F 5'-GGCTGTGGGCAAGGTCATCCCTGA-3'; primer R 5'-TCCACCACCCCTGTTGCTGA-3'). Quantitative Real Time PCR (qRT-PCR) amplification was performed using Fast SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). cDNA solution (1 μl) was amplified in a 10 μl solution containing 2X Fast SYBR Green Master Mix, 0.3 pmol of each primer and DEPC water to reach the final volume. Primer sets for qRT-PCR, showed in the following table (Table 2), were designed using Primer3 tool.

<table>
<thead>
<tr>
<th>Human genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1</td>
<td>5'-TGGAAGTCAACCCACGCTATC-3'</td>
<td>5'-TCGCAAGGTGCTGCTTGG-3'</td>
</tr>
<tr>
<td>FLI1</td>
<td>5'-ATAGACCACACAGGAGGAG-3'</td>
<td>5'-GTCGCTGCCCATCCTATC-3'</td>
</tr>
<tr>
<td>KCNJ1</td>
<td>5'-TGGACATCTGGACAACGGTA-3'</td>
<td>5'-CACACAGGGAGTGTGATTGG-3'</td>
</tr>
<tr>
<td>KCNJ5</td>
<td>5'-GTCCTGGCCATCCATGTC-3'</td>
<td>5'-GAGAACAGGAAAGCGGACAC-3'</td>
</tr>
<tr>
<td>TPS3AIP1</td>
<td>5'-ACCAGAACCTCCTCCGATG-3'</td>
<td>5'-TACCTGGTGCTCGTGATG-3'</td>
</tr>
<tr>
<td>ARHGAP32</td>
<td>5'-ACATCCATCTTGGGGTCC-3'</td>
<td>5'-GCTGGAGCACATCTGAGGAT-3'</td>
</tr>
<tr>
<td>BCL2</td>
<td>5'-AGTACATCAGGACACCAAT-3'</td>
<td>5'-TCATTAGGGTGACGATTG-3'</td>
</tr>
<tr>
<td>PAX5</td>
<td>5'-GGCAAAGAGAGCCAGACG-3'</td>
<td>5'-GCCCTGTGCTGCTGAGAT-3'</td>
</tr>
<tr>
<td>BCL6</td>
<td>5'-ATGGAGCCTGGAACTCCATG-3'</td>
<td>5'-GGGTGACATGAGGATGGA-3'</td>
</tr>
<tr>
<td>PRDM1</td>
<td>5'-ACATGGACCCAGGACACTCA-3'</td>
<td>5'-GGCACTGATGAGGATGGA-3'</td>
</tr>
<tr>
<td>XBP1</td>
<td>5'-TAGACAGCCTGACAGTCGAGA-3'</td>
<td>5'-CCAAGAGCGCTGCTTAACTTC-3'</td>
</tr>
<tr>
<td>IRF4</td>
<td>5'-AAGGATAAAGGCCTGGCAAG-3'</td>
<td>5'-TTTTCCTGCGCCAGTGGC-3'</td>
</tr>
<tr>
<td>CXCR5</td>
<td>5'-TACGGTGCTGAAATGACCCT-3'</td>
<td>5'-CAGGAGGAAGATGAGGCTG-3'</td>
</tr>
<tr>
<td>CXCR4</td>
<td>5'-TGGCCGGGCAACTGCTACT-3'</td>
<td>5'-GAGCCGCAATGACGACACTG-3'</td>
</tr>
<tr>
<td>CXCR7</td>
<td>5'-GTCCTGGCTCAACATGAGA-3'</td>
<td>5'-TGTGGCTTGCTGCTGACGT-3'</td>
</tr>
<tr>
<td>NF-KB1</td>
<td>5'-GAGAGATGGGATTGCCCTG-3'</td>
<td>5'-TCGCTGGCTGCGCTAGT-3'</td>
</tr>
<tr>
<td>CDC25B</td>
<td>5'-TTCATCAGGGAGAGGAGGAG-3'</td>
<td>5'-AGGCCGGAAGGTCTTACCTG-3'</td>
</tr>
<tr>
<td>WEE1</td>
<td>5'-TGAAGACATGGGAGGAGCCAGT-3'</td>
<td>5'-ATCCATCAGGGCTTCTACA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAGGCTGAGGAAAGAGGAG-3'</td>
<td>5'-CCCCGTGGTCTGACGCAAAT-3'</td>
</tr>
<tr>
<td>β2m</td>
<td>5'-TATCCAGCGTACTCCAAGA-3'</td>
<td>5'-GACAAGTGCTGAATGCTCCAC-3'</td>
</tr>
</tbody>
</table>

Table 2: primers list.

Polymerase chain reactions were performed using a 96-wells plate and optical adhesive covers (Applied Biosystem). The following program was run on the thermal cycler: 95°C
for 15’, followed by 40 cycles of 95°C for 15’ and 60°C for 1’. All samples were analyzed in triplicates. A no-template control was added to each plate and for each assay, and contained all PCR reagents without any template DNA. The relative quantity of the specific mRNA for each sample was calculated based on mean Ct values using the delta-delta Ct with a correction for experimental variations by normalization to the housekeeping gene GAPDH.

**WESTER BLOTTING (WB)**

Cells were solubilized in hot SDS lysis buffer (2.5% SDS, Tris-Hcl pH7.4) and sonicated for 15”. The protein concentration in each sample was determined using the BCA protein assay (Pierce Chemical Co.) that, based on bicinchoninic acid, enables a colorimetric detection and quantification of total protein. The method combined the reduction of Cu²⁺ to Cu⁺ by proteins in alkaline medium with the colorimetric detection of Cu⁺ and a reagent containing BCA. The chelation of two molecules of BCA with one cuprous cation (Cu+) gives a purple coloration and the complex strongly absorbs at 570 nm. Absorbance can be detected by 96-well plate reader (Beckman Coulter AD340), and it is directly proportional to the protein concentration. Lysates (20 μg) were fractionated by SDS-PAGE using 8%-10% polyacrylamide gels, based upon the expected molecular weight. The resolved proteins were blotted to a nitrocellulose membrane by electric transfer, and the membranes were blocked in 0.2% I-Block in PBS for one hour at RT. Membranes were incubated overnight at 4°C with primary antibodies prepared in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.5% Tween 20) according to optimized dilutions. The following primary antibodies were used: anti-ETS1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLI1 (C-19, Santa Cruz Biotechnology), anti-PRDM1a (6D3, Santa Cruz Biotechnology) and anti-GAPDH (MAB374, Millipore, Zug, Switzerland), anti-α-tubulin (C-11, Santa Cruz Biotechnology, Santa Cruz, CA) anti-IRF4 (no. 4964, Cell Signaling technology), and anti-XBP1s (clone 143F, Biolegend, CNIO, Spain). Membranes were washed in TBS-T three times for 10’ each and then incubated in TBS-T containing the appropriate horseradish peroxidase-conjugated anti-mouse secondary antibodies (Amersham Life Science) for one hour at RT. The membranes were washed three times for 10’ each in TBS-T and then processed for enhanced chemiluminescence detection according to the manufacturer’s instructions (ECL; Amersham Life Science). Equal loading of samples was confirmed by probing for GAPDH.
CHROMATIN IMMUNOPRECIPITATION (ChIP)

For BLIMP1 promoter detection, OCI-LY7 cells were previously infected with shGFP and shETS1 and selected for 72h. For CXCR5 promoter detection OCI-LY7 and SUDHL-2 were not previously treated. Cells were cross-linked with formaldehyde to a final concentration of 1% for 10' at RT. 1 ml of glycine 10X was added and cells were incubated for 5' at RT. Cells were then moved in ice for 1' and centrifuge at 1200 rpm for 10'. After two washes with PBS 1X, 5 μl of Protein Inhibitor Cocktail (PIC) in 1 ml of PBS 1X were added to cell pellet. Cells were then lysed in 1 ml of lysis buffer plus 5 μl of PIC and sonicated. After sonication, 10'' x 10 times on ice waiting 20'' between each sonications, aliquots of 100 μl were prepared. After a treatment with agarose G chromatin was immunoprecipitated over-night with antibody for ETS1 (C-20 X, Santa Cruz Biotechnology). After a second passage with agarose G, immunoprecipitated samples were washed and eluted. Finally, DNA-protein cross-links were reversed and DNA purified from total cell lysates (input) and immunoprecipitated fractions. qRT-PCR or standards PCR were performed using the following primers: PRDM1 FW 5'-GAGAAGCAGGAATG CAAGGT-3', PRDM1 REV 5'-AGCGGTCGGAGGCAGTAAT-3', CXCR5 FW 5'- AGAGACATGGTCTTGCTATGT-3' and CXCR5 REV 5'- CAACAGAGGCAAAACCCCTTC-3'. The amount of immunoprecipitated DNA was calculated in reference to a standard curve and normalized to input DNA.

6. GENE EXPRESSION

GENE EXPRESSION PROFILING (GEP)

GEP was performed after single ETS1 and FLI1 down- regulation using both shRNAs against each gene in two or three independent experiments. Total RNA was isolated using Trizol (Invitrogen Life Technologies, Carlsband, CA, USA). The concentration and the quality of total RNA were assessed with NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA).

Illumina Whole-Genome Gene Expression BeadChip consists of oligonucleotides immobilized to beads held in microwells on the surface of an array substrate (Fig.13). Each array on the Human HT-12 v4 Expression BeadChip targets more than 47,000 probes derived from the NCBI RefSeq Release 38 (November 7, 2009).

500 ng of RNA starting material was amplified using a single-round in vitro transcription (IVT) amplification with the Illumina TotalPrep RNA Amplification Kit (Ambion). The Illumina RNA Amplification Kit is a complete system for generating biotinylated and amplified
RNA for hybridization using reverse transcription followed by a T7 RNA polymerase-based linear amplification step. Briefly, 11 μl of RNA appropriately diluted in water, were mixed with a Reverse Transcription Master Mix, containing T7 Oligo(dT) primer, 10X First Strand Buffer, dNTP Mix, RNase Inhibitor and Array Script reaching the final volume of 20 μl. The reaction was incubated at 42°C for two hours (Fig.14).

A Second Strand Master Mix was prepared with Nuclease-free Water, 10X Second Strand Buffer, dNTP Mix, DNA Polymerase and RNase H. 80 μl of the second mix were transferred to each sample. Tubes were placed in a thermal cycler and were incubated at 16°C for two hours. Then the reactions were placed on ice and the cDNA obtained was purified. We added 250 μl of cDNA Binding Buffer to each sample and mixed the reaction which was then pipetted on the centre of cDNA Filter Cartridge. After having centrifuged them and washed the cDNA Filter Cartridge, the cDNA was eluted with 20 μl of 55°C pre-heated Nuclease-free Water. The cDNA sample was transferred in a PCR tube and 7.5 μl of IVT Master Mix (T7 10X Reaction Buffer, T7 Enzyme Mix and Biotin-NTP MIX) were added to cDNA. Once assembled, tubes were placed in a thermal cycler at 37°C for 14 hours. The reaction was stopped by adding 75 μl of Nuclease-free Water to each cRNA sample to bring the final volume to 100 μl. The cRNA purification was performed adding 350 ml of cRNA Binding Buffer to each cRNA sample. 250 μl of ACS reagent grade 100% ethanol were added to each sample and each mixture was pipetted onto the center of the filter in the cRNA Filter Cartridge. After centrifugation and a washing of the filter, cRNA was eluted with 200 μl of 55°C pre-heated Nuclease-free Water.

**Figure 13:** Beads immobilize each oligonucleotide that binds to the probe on BeadChip. Figure adapted from Illumina TotalPrep RNA Amplification Kit, Protocol.

The next step forecast the cRNA hybridization. The cRNA samples were preheated at 65°C for 5’. After they have been cooled, the appropriate volume of cRNA for each sample was added to Hyb Mix and RNase-free Water and the mixture was hybridized to
the BeadChip. The Human HT-12 v4 Expression BeadChip content provides genome-wide transcriptional coverage of well-characterized genes, gene candidates, and splice variants, delivering high-throughput processing of two samples per BeadChip.

To allow the hybridization of cRNA to the BeadChip, the latter one was placed into the 58°C Illumina Hybridization Oven for 14 hours. Washings were performed in Hybex

Waterbath as described in the Whole-Genome Gene Expression Direct Hybridization Assay Guide. In the end, BeadChips were placed in a Wash Tray where Block E1 buffer was added, and after 10', they were washed and dried. In the last step, Cy3-SA is introduced to bind to the analytical probes that have been hybridized to the BeadChip.
This allows for differential detection of signals when the BeadChips are scanned. The iScan Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections.

Arrays were read on an Illumina HiScanSQ system. Data were first extracted with the Illumina GenomeStudio software and then imported in Partek Genomics Suite 6.4.

GEP data were analyzed using Partek Genomic Suite v. (Partek). Signal intensities were quantile normalized. Transcripts with differences in expression were identified by ANOVA (statistical analysis of the variance) using both shRNA for each gene and the control shGFP. A gene list was generated considering as significant genes with an absolute fold change >1.2 and FDR <0.20.

**GENE SET ENRICHMENT ANALYSIS (GSEA)**

Functional annotation was performed using the Gene Set Enrichment Analysis [125]. Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological state (http://www.broadinstitute.org/gsea/index.jsp). The method derives its power by focusing on gene sets, that is, groups of genes (pathways) that share common biological function, chromosomal location, or regulation, and allows to identify deregulated pathways after specific perturbations. We compared the gene expression levels from cells infected with both shRNA against each genes and cells infected with the control shGFP and picked up the genes which had significant different expression for GSEA by using Molecular Signatures Database (V3.0). GSEA was carried out by computing overlaps with canonical pathways (CP) and chemical and genomic perturbations (CGP) obtained from the Broad Institute. Raw data will be available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database.
Results
Results

**PRIMARY AIM**

Part of the data reported in the following section have been published as: Bonetti P*, Testoni M* et al. Deregulation of ETS1 and FLI1 contributes to the pathogenesis of Diffuse Large B-Cell Lymphoma. Blood 2013;122 (13): 2233-2241.

*, equally contributed

1. CHARACTERIZATION OF A NEW RECURRENT GAIN AT 11q24.3 GENOMIC LOCUS OF DLBCL PATIENTS CONTAINING TWO INTERESTING ETS FACTORS

1.1. 11q24.3 GAINS A RECURRENT LESION IN A SUBSET OF DLBCL PATIENTS

A previous work of our group had analyzed DNA genome profiles of a series of 166 DLBCL patients [186]. The analysis of genomic profile by high-density genome-wide SNP-based array [186] had identified a minimal common region (MCR) of gain at 11q24.3 not previously functionally characterized, in 23% (31/166) of the cases.

[Figure 15]: upper panel: copy number frequency plot of whole genome of 166 DLBCL samples. Gains are represented in red, losses in blue. X-axis, represents all chromosomes and their physical mapping; Y-axis, represents the frequency of patients affected by alterations. Lower panel: Frequency of gains on chromosome 11 alone in 166 DLBCL samples. X-axis, physical mapping; Y-axis, frequency of patients with gains. 11q24.3 gain is indicated by the black arrow [186].
Results

Figure 16: Genes localized in 11q.24.3 locus according to the UCSC data base (NCBI Build 36.1).

Figure 15 (upper panel) represents the frequency plot of detected DNA gains and DNA losses.

The genome of DLBCL patients is characterized by a high complexity with a lot of aberrations that vary from gains and amplifications to losses and deletions that confirms the difficulty in classification and treatment of DLBCL cases. This analysis is not used to identify translocations.

It was decided to study deeper the region at chromosome locus 11q24.3 (GRCh37/hg19chr:128 137,4000-128 994,334, Fig.15 lower panel) not only for the frequency of the gain, but also because chromosome 11q is very genes rich and it is the target of a variety of chromosomal aberrations in different hematological cancers. This aberration contain 6 transcripts: ETS1, FLI1, KCNJ1, KCNJ5, P53AIP1, and RICS (Fig.16).

1.2. ETS1 AND FLI1 ARE THE TWO TARGETS OF 11q24.3 GAIN

Integration of genomic profiles with gene expression analysis done with the Affymetrix U133 plus 2.0 on a set of 54 cases (16 bearing 11q24.3 gain) showed that, among the six genes previously described, only the ETS1 and FLI1 genes were expressed in DLBCL patients (Fig.17). Moreover, cases with 11q24.3 gain presented significantly higher RNA levels of ETS1 (fold change: 1.4; P=0.0129) and FLI1 (fold change: 1.5; P=0.0082) than wild type cases suggesting that in these cases the gain affects the expression of the two factors (Fig.17). Correlation analysis with gene expression data revealed that high levels of ETS1 mRNA correspond to high FLI1 mRNA levels pointing out that patients carrying the 11q24.3 gain express concomitantly high levels of ETS1 and FLI1 (correlation coefficient 0.73; Fig.18).
Results

Figure 17: 11q24.3 gain is a recurrent event in DLBCL and it is associated with high levels of ETS1 and FLI1. Patients with the 11q24.3 gain express high levels of ETS1 and FLI1 mRNA. RNA levels of ETS1, FLI1, KCNJ1, KCNJ5, TP53AIP1, and RICS in patients with 11q24.3 gain compared with patients without the gain (wt). *P value <0.05.

Figure 18: Correlation between ETS1 and FLI1 mRNA levels in DLBCL samples. Values of mRNA major then 7 correspond to the expression status of the gene. Here just one samples does not expresses ETS1 and FLI1.
Results

Figure 19: Immunohistochemical analysis of ETS1 and FLI1 in three representative DLBCL cases carrying the 11q24.3 gain (40X).

Using immunohistochemical analysis, expression of ETS1 and FLI1 was also confirmed at protein level on 12 DLBCL cases, 4 bearing the gain (Fig.19). The same analysis was used to detect the expression of ETS1 and FLI1 in normal B cells from human tonsil (Fig.20). Here, the Ki-67 marker, a protein strictly associated with cell proliferation, was used to distinguish dark proliferative zone of centroblasts to the light non-proliferative zone of centrocytes. We observed that ETS1 and FLI1 are both expressed with an expression gradient from dark to light zone, with a stronger difference for FLI1. This finding confirms previous knowledge from the literature in which ETS1 and FLI1 are essential factors for early phases of GC. As we can see, other important lymphatic cells such as macrophages, dendritic cells or T cells around the GC, also express ETS1 and FLI1.

Because DLBCL molecular subtypes are characterized by specific chromosomal aberrations, I try to understand if 11q24.3 gain is associated with one DLBCL subtypes, but no significant associations were revealed by this analysis. A correlation emerged by GEP analysis using GSE10846 dataset [161], in which the mRNA levels of both ETS factors derived from ABC- and GCB-DLBCL patients (n=167; n= 183, respectively) and from hyperplasia samples (9 cases). ETS1 levels were higher in ABC- and GCB-DLBCL cases than in benign hyperplasia (P=.013 and P =.035, respectively) and in ABC- than in GCB-
Results

DLBCL (P<0.001), suggesting that their expression can have a role in lymphomagenesis, in particular, in maintaining the early phase conditions of GC (Fig.21 left panel). FLI1 expression levels did not show any differences between the two DLBCL subtypes or in respect to the benign hyperplasia. ETS1 was more expressed in normal B cells compared to FLI1 (Fig.21 right panel), thus, maybe, a variation in ETS1 expression is more detectable than variations in FLI1 mRNA levels.

![Figure 20: Immunohistochemistry on normal human tonsil using antibodies anti-Ki67, ETS1 and FLI1. CB=centroblasts, CC=centrocytes.](image1)

![Figure 21: Left panel: GEP analysis on ABC-/GCB- DLBCL samples and hyperplasia samples to detect ETS1 and FLI1 expression differences. GEP data from LLMPP DLBCL series vs Hyperplasia. Right panel: qRT-PCR for ETS1 and FLI1 detection on a pool of cDNA from naïve B cells, centroblasts, centrocytes, memory B cells. mRNAs from B cells of different origin were join together during qRT-PCR.](image2)
2. FUNCTIONAL ANALYSIS OF ETS1 AND FLI1 IN A DLBCL CELL LINE CARRING THE GAIN.

2.1. OCI-LY7 IS THE BEST DLBCL CELL LINE TO STUDY THE ROLE OF ETS1 AND FLI1

To study ETS1 and FLI1 role in DLBCL pathogenesis, I looked for a cellular system, taking advantage of the numerous DLBCL cell lines available in our laboratory. To find out a suitable lymphoma cell line I screened all the DLBCL cell lines available, both GCB and ABC subtype, and I observed that, generally, as ETS1 as FLI1 are expressed at moderate high levels in almost all DLBCL cell lines (Fig.22 upper panel). Among all, OCI-LY7 cell line appeared the best cellular model for two reasons: 1. OCI-LY7 expresses the highest amount of ETS1 and good levels of FLI1 proteins. 2. Genomic profile analysis revealed that OCI-LY7 cell line carries the exact gain at 11q24.3 observed in clinical samples (Fig.22 lower panel).

![Figure 22](image-url)

Figure 22: WB analysis on 22 DLBCL cell lines. ETS1 and FLI1 protein levels were determined by immunoblotting of whole cell lysate; GAPDH was used as loading control. Blue: GCB subtype DLBCL Red: ABC subtype DLBCL. Lower panel: genomic profile analysis on OCI-LY7. Representation of a small part of chromosome 11q with the gain at 11q24.3 locus. The gain is indicated with a blue circle.
2.2. ETS1 AND FLI1 DOWN-REGULATION USING DIFFERENT METHODS

A series of different transfection analyses with siRNA against ETS1 and FLI1 were performed. At first, I set up transfection experiment using 50nM and 70nM of lipofectamine and siRNA against ETS1, FLI1 and the control sample GL3 using the same lipofectamine rate. Transfection efficiency was determined at 48, 72 or 96 hours, but from all the preliminary experiments I did not obtain ETS1 or FLI1 down-regulation (Fig. 23). I also tried to perform a double transfection with lipofectamine using 50nM of both siRNA to obtain a stronger down-regulation of ETS1 and FLI1 genes. After 48h, cells were transfected again using the same conditions. However, gene silencing was not obtained (Fig. 24).

**Figure 23:** Western Blot analysis after 96h of transient transfection with lipofectamine in OCI-LY7 DLBCL cell line. Left panel: FLI1 protein levels in OCI-LY7 transfected with 50nM or 70nM of siRNA against FLI1. Right panel: ETS1 protein levels in OCI-LY7 transfected with 50nM or 70nM of siRNA against ETS1. α-tubulin was used as loading control. CTR: samples treated only with Optimem medium. LIPO: samples without siRNA addition. siFLI1 and siETS1: samples transfected with 50nM or 70nM of each siRNA to obtained the specific FLI1 or ETS1 down-regulation. siGL3: scramble control.

**Figure 24:** Western Blot analysis using antibodies against FLI1, ETS1 and α-tubulin after 48h from the second transfection with lipofectamine in OCI-LY7 DLBCL cell line. siRNA were used 50nM concentrated.
Then, for its higher transfection efficiency and low cell mortality, electroporation method using BIO-RAD Gene Pulser II electroporator machine was used to transfect OCI-LY7 cell line. Cells were electroporated at 250V with siRNAs against each ETS factors and GL3 scramble control. Again, with this approach any down-regulation of ETS1 and FLI1 was obtained (Fig.25).

**Figure 25:** Regular PCR analysis of RNA samples extracted from OCI-LY7 treated with siRNA against FLI1 or ETS1 expression. RNA was extracted after 96h from electroporation treatment. Expression of FLI1 and ETS1 was evaluated together with the housekeeping, GAPDH. As scramble control was used siRNA against GL3 mRNA. CTR: sample electroporated without siRNA addition.

ETS1 and FLI1 stable silencing was obtained only with lentivirus strategy. The shRNA construct used was an HIV-based lentiviral vector (pLKO.1), which contains two antibiotic resistance markers (puromycin mammalian selectable marker, and Ampicillin bacterial selectable marker) (Fig.26).

**Figure 26:** The pLKO.1 vector. The shRNA construct are designed to include a hairpin of 21 base pair sense and antisense stem and 6 base pair loop (Xhol restriction site: CTCGAG). Human U6 promoter is generated with four uridine at each 3’ end. hPGK: Human phosphoglycerate kinase promoter. sinLTR: 3’ Self inactivating long terminal repeat. 5’LTR: 5’ long terminal repeat. RRE: Rev response element. cPP: central polyuridine tract. f1 ori: f1 origin of replication. Puro and amp are the two resistance selectable markers, mammalian and bacterial respectively (modified by Open Biosystems).
Self-inactivating replication incompetent viral particles containing shRNA for ETS1, FLI1 or GFP scramble control, were produced by cotransfection of HEK-293T packaging cells with pLKO.1 transfer vector, pCMV-dR8.74 packaging vector and pMD2.VSVG envelope vector (third-generation helper plasmids).

The selection of infected cells was performed using the acquired resistance to puromycin, at dose selection of 0.4 µg/ml for 72 hours. To allow lentivirus infection we used the cationic polymer polybrene (hexadimethrine bromide) at a concentration of 6 µg/ml, not toxic for OCI-LY7 cell line. This polymer acts by neutralizing the charge repulsion between virions and sialic acid on the cell surface allowing the lentivirus to enter into cells.

For both ETS1 and FLI1 genes I first tested five different hairpins (sh60A/B/C/D/E against ETS1 and sh61A/B/C/D/E against FLI1) and, for each gene, the best two hairpins were selected (sh60A and sh60D for ETS1 and sh61C and sh61E for FLI1). Down-regulation of ETS1 and FLI1 was evaluated by WB and qRT-PCR analysis. Both ETS1 and FLI1 were efficiently down-regulated, although not reaching a complete silencing of genes (Fig.27). Hence, in the text the names shETS1 and shFLI1 alone, without specify the kind of vector, will refer to sh60A and sh61E respectively, the shRNAs that worked better against each genes, as shown by WB analysis (Fig.27).

**Figure 27:** ETS1 and FLI1 down-regulation in OCI-LY7 cell line. ETS1 and FLI1 protein levels were determined by immunoblotting of whole cell lysate; GAPDH was used as loading control. ETS1 and FLI1 mRNA levels were determined by qRT-PCR; B2microglobulin was used as housekeeping gene. shGFP is used as control, sh60A and sh60D are two shRNAs that target ETS1, sh61C and sh61E target FLI1.
2.3. ETS1 AND FLI1 SUSTAIN OCI-LY7 CELL VIABILITY AND PROLIFERATION

Being that the most dangerous characteristics of a tumor are uncontrolled cell proliferation and cell survival, I examined the possible role of ETS1 and FLI1 down-regulation on these two phenotypic effects in OCI-LY7 cell line.

To do this, I performed a growth curve assay in which OCI-LY7 were seeded in triplicate on 24-well plates at a density of 3x10^5 cells/well. Cultures were harvested every day, and the number of cells was determined. I found that both ETS1 and FLI1 down-regulation induced a lower proliferation rate compared with the shGFP control (Fig.28 left panel).

Further analysis revealed that this phenotype is associated with a reduced percentage of cells in S-phase. To see this effect I performed at day 6 an EdU assay. Cells were treated with EdU molecule, a nucleotide analog to thymidine that is incorporated into DNA during active DNA synthesis (Fig.28 right panel).

![growth curve](image1)

**Figure 28:** Left panel: growth curve, of OCI-Ly7 cells infected with control (shGFP) lentivirus or a lentivirus expressing shRNA for ETS1 and FLI1 (shETS1 and shFLI1, respectively). Right panel: levels of EdU incorporation were determined by FACS analysis. Percentage refers to cell in an active S-phase. EdU-Alexa-488 detected with FL1-H channel.

To evaluate the biological effects of ETS1 and FLI1 down-regulation at longer time points, a co-culture experiment was performed in which cells infected with shRNA against ETS1 or FLI1 (GFP negative) were added to an equal amount of cells infected with a vector carrying the GFP marker (GFP positive). FACS analysis of the percentage of the two cell populations at days 3, 7, 10, 17, and 24 following infection demonstrated that OCI-LY7 cells with reduced levels of ETS1 or FLI1 proliferated less efficiently and were outgrown by GFP positive cells thereby indicating an impairment of cell proliferation as a result of the ETS1 or FLI1 down-regulation (Fig.29). Control shGFP transduced cells did not display this readout. In comparison with shETS1 cells, shFLI1 cells were more rapidly overcome by
GFP-positive cells despite a similar percentage of regulation for both proteins, suggesting a more prominent role for FLI1 in regulating cell proliferation.

Figure 29: Evaluation of cell proliferation after double-infection. Infected cells (GFP -, light red bars) with shRNA against GFP, ETS1, FLI1 and both ETS1 and FLI1, two days after infection have been mixed 1:1 with OCI-LY7 previously infected with a GFP expressing vector (GFP+, blue bars) and percentage of GFP positive cells have been evaluated through FACS analysis at days 3, 7, 10, 17 and 24.

In order to investigate whether this phenotype was associated with cell death annexinV-FITC/PI staining was performed, treating cells four days after infection. Both hairpins against ETS1 and FLI1 induced apoptosis in a significant percentage of OCI-LY7 (Fig.30), indicating that ETS1 and FLI1 are required for cell viability in OCI-LY7.

Figure 30: ETS1 (upper) and FLI1 (lower) down-regulation induces apoptosis. Percentage of apoptotic cells was determined by AnnexinV staining after four days of infection. Dot plots are representative of one experiment; histogram graphs represent the average of at least 3 independent experiments. *P value < 0.05.
Finally, because ETS1 and FLI1 are very close by mapped and since they were found concomitantly expressed in DLBCL patients, I investigated whether ETS1 and FLI1 could cooperate in regulating cell viability. A successive down-regulation was performed using shRNA against GFP and ETS1 for the first infection, because of the lower mortality obtained after their use compared to the mortality rate after FLI1 down-regulation. Then I re-infected cells with shRNA against FLI1. For all conditions cells were infected twice. For single silencing, cells were transduced with the shRNA against GFP and with the shRNAs against either of the two ETS factors. For control condition cells were treated twice with shGFP. The down-regulation of ETS1 and FLI1 genes was detected by qRT-PCR and WB analysis (Fig. 31).

**Figure 31:** ETS1 and FLI1 concomitant down-regulation in OCI-Ly7 cell line. qRT-PCR (left) and WB (right) analysis of ETS1 and FLI1 levels after four days of infection.

Concomitant down-regulation of ETS1 and FLI1 clearly induced apoptosis in a higher percentage of cells (70%) (Fig. 32) respect single transduced cells (Fig. 30). This strongly indicated that ETS1 and FLI1 could cooperatively sustain cell viability in DLBCL patients.

**Figure 32:** Evaluation of apoptosis after double-infection. Dot plots are representative of one exemplary experiment. Histogram plots represents the average of three independent experiments. 

** p<0.05
3. ETS1 AND FLI1 DOWN-REGULATION IN OTHER DLBCL CELL LINES

To assess whether ETS1 and FLI1 are generally required for cell viability in primary DLBCL independently on the presence of the 11q24.3 gain and high levels of ETS1 and FLI1 expression, I down-regulated the expression of these two molecules in a set of GCB- (SUDHL-4, VAL) and ABC- (OCI-LY10, SUDHL-2, and U-2932) DLBCL cell lines lacking the 11q24.3 gain. The down-regulation of ETS1 and FLI1 genes in these DLBCL cell lines was detected by qRT-PCR and WB analysis (Fig.33). I used DLBCL cell lines associated to both DLBCL GEP signatures in order to see if phenotypic effects deriving from ETS1 and FLI1 silencing were associated with the differ molecular subtypes.

![Figure 33: ETS1 and FLI1 down-regulation in SUDHL-4, VAL, U2932, SUDHL-2, and OCI-LY10 DLBCL cell lines. qRT-PCR (upper panel) and WB (lower panel) analysis of ETS1 and FLI1 levels after shRNA (day 5). In black are shown GCB-DLBCL cell lines, in red are shown ABC-DLBCL cell lines. As a control, β2-microglobulin mRNA and GAPDH protein expression was documented.](image)

The staining of these DLBCL infected cells with annexin V showed that only FLI1 down-regulation significantly affected cell viability in all DLBCL cell lines similarly to FLI1-dependent mortality that occur in OCI-LY7, whereas ETS1 down-regulation did not induce apoptosis in the same cell lines (Fig.34). In contrary, ETS1 down-regulation strongly affects cell proliferation as happen for FLI1 silencing (Fig.35) suggesting that the two
genes are active on different pathways in these DLBCL models. Notably, in the majority of the DLBCL cell lines, ETS1 down-regulation induced a significant FLI1 up-regulation (Fig.33) suggesting that elevated FLI1 levels might compensate for ETS1 down-regulation in term of cell death.

Figure 34: Annexin V analysis on 5 different DLBCL cell lines after ETS1 and FLI1 down-regulation. Percentage of apoptotic cells was determined after 5 days of infection. Histogram graphs represent the average of at least two independent experiments.*P value <0.05.

Figure 35: Growth curve, of SUDHL-4 and SUDHL-2 DLBCL cell lines infected with control (shGFP) lentivirus or lentivirus expressing shRNA against ETS1 and FLI1.
**4. ANALYSIS OF ETS1 AND FLI1 TARGETS IN DLBCL CELL LINES**

**4.1. ETS1 REGULATES GENES INVOLVED IN B CELL DIFFERENTIATION IN OCI-LY7 DLBCL CELL LINE**

Considering that both ETS1 and FLI1 genes encode for transcription factors and that there are evidences suggesting their involvement in B cell development, I was interested in studying whether ETS1 and/or FLI1 would, directly or indirectly, regulate genes known to be crucial for GC regulation. Within this aim, I evaluated, by qRT-PCR, a series of genes known to play a role in GC B cells after silencing of FLI1 and ETS1 with the two shRNA against each genes. The evaluated genes were: BCL2, PAX5, BCL6, PRDM1, XBP1 and IRF4 (Fig.36).

I observed that ETS1 down-regulation results in a significant up-regulation of PRDM1 and XBP1 acting downstream to PRDM1, and this effect was maintained even when both ETS factors were down-regulated (Fig.37). Interestingly, the down-regulation of PRDM1 and XBP1 occurs with no effect on IRF4 (Fig.37), suggesting that ETS1 might act upstream or independently from the NF-kB pathway. These data were also confirmed at protein level (Fig.37).
Results

Figure 37: Left panel: RNA levels of PRDM1, XBP1 and IRF4 were determined by qRT-PCR after concomitant ETS1 and FLI1 down-regulation (day 3). b2-microglobulin was used as the housekeeper. *P value <0.05. Right panel: western blot analysis with antibodies against ETS1, PRDM1, IRF4, active splice form XBP1s, and GAPDH as loading control after shETS1.

PRDM1 encodes for BLIMP1, thus I refer to BLIMP1 only for the protein product of the gene. For a further confirmation of these data, I performed a rescue experiment in which OCI-LY7 cells were previously interfered for ETS1 expression using lentiviral infection method and were leave in selection for 72 hours. Then, I introduced pCMV6-ETS1 expression vector in OCI-LY7 through electroporation (amaxa optimization nucleofector method), to obtain a good re-expression of the gene. After 24 hours I collected RNA samples and observed that ETS1 reintroduction restored PRDM1 mRNA levels to values similar to the control cells (shGFP) indicating that the observed PRDM1 up-regulation was induced by ETS1 silencing (Fig.38).

Figure 38: Left panel: rescue experiment for ETS1 expression. ETS1 reintroduction restores PRDM1 mRNA levels in OCI-Ly7. RNA levels of ETS1 and PRDM1 were determined by qRT-PCR at 24 hours after ETS1 re-expression in cells previously interfered for ETS1 expression. *P value <0.05.
In order to evaluate whether PRDM1 was directly regulated by ETS1, I looked for the presence of ETS binding sites (EBS) on PRDM1 promoter and I found a canonical EBS (21035 bp). Then a Chromatin Immunoprecipitation (ChIP) was performed in order to assess the direct binding of ETS1 to the EBSs within PRDM1 promoter. With this assay DNA-protein complexes are detected by the isolation of DNA using antibodies recognizing specific proteins directly bound to promoter sequences. To isolate ETS1-DNA immunocomplexes, antibodies against ETS1 protein were used on total DNA from OCI-LY7 cells previously infected with the control vector shGFP and the shETS1 as shown by WB analysis (Fig.39). For qRT-PCR I used a pair of primers specific for flanking sequences of one of the putative EBSs inside PRDM1 promoter. We detected an enrichment of PRDM1 promoter region containing the EBS in samples without ETS1 silencing (control samples infected with shGFP). Enrichment was reduced when ETS1 expression was down-regulated (Fig.39). I can thus conclude that ETS1 negatively regulates PRDM1 transcription in a direct manner. Unfortunately, I was not able to discover any FLI1 specific targets genes also because many of its known targets are affected by genomic aberrations in DLBCL cell lines (e.g. c-MYC that is rearranged in OCI-LY7).

Figure 39: ChIP analysis of PRDM1 promoter performed with antibody against ETS1 in OCI-LY7 infected with the control shGFP or shETS1 lentivirus. Input and immunoprecipitated DNA was amplified by qRT-PCR using primers amplifying the D-1100/-939 bp region of the PRDM1 promoter. Enrichments are presented as percentage of total input DNA.

To understand if the molecular effect of ETS1 on PRDM1 gene depends on the presence of the gain, PRDM1 levels were analyzed after ETS1 knock-down in others DLBCL cell lines that did not carry the 11q24.3 gain. I evaluated PRDM1 levels in SUDHL-4, SUDHL-2 and U2932 DLBCL cell lines after infection with pLKO.1 lentiviral vectors containing shRNA against GFP and ETS1. I did not observed an up-regulation of PRDM1 expression (Fig.40).
Results

This effect is due for genomic alterations that affect PRDM1 gene in all these DLBCL cell lines. SUDHL-4 is dependent from BCL6 expression. SUDHL-2 has PRDM1 silencing mutation, while U2932 has PRDM1 loss. Thus, up to now I was not able to detect the up-regulation of PRDM1 in a different DLBCL cell line model. One possibility could be to use Toledo, a GCB-DLBCL cell line in which BCL6 is not expressed.

Figure 40: ETS1 down-regulation detected by qRT-PCR analysis in SUDHL-4, SUDHL-2 and U2932 DLBCL cell lines. b2-microglobulin was used as the housekeeper.

Further experiments were performed in order to better understand the biological effects of the regulation of PRDM1 gene by ETS1. These experiments were done without success. At first, I tried to understand if the phenotypic effect of ETS1 in OCI-LY7 cell line was due to the down-regulation of ETS1 itself or by the up-regulation of PRDM1, known to be a tumor-suppressor gene [222, 343, 344]. I performed infections with OCI-LY7 using PWPI-expression vectors, one containing the entire PRDM1 gene linked to an HA tag, and one representing the empty version of the vector. PWPI vector contains also the GFP expression marker to allow the sorting of infected cells. PWPI is a bicistronic vector in which GFP and HA-PRDM1 genes were under the control of the same EF1-alpha promoter. As for pLKO.1, PWPI lentiviral particles were produced by cotransfection of HEK-293T cells with pCMV-dR8.74 packaging vector and pMD2.VSVG envelope vector (third-generation helper plasmids).

Having GFP marker, I evaluated the percentage of PWPI-infected cells through FACS analysis after 24, 48 and 72 hours without obtained a good percentage of transduction but obtaining a very high mortality. I thought that the unsuccessful experimental could be explained in two ways: 1. The PWPI vector is toxic for OCI-LY7 cells because of its size
Results

(11076 bp). A possible solution could be to infect cells with a lower amount of PWPI-expressing vector obtaining a lower percentage of infected cells but maybe also lower infection toxicity. 2. HA-BLIMP1-IRES-GFP bicistronic gene was under the control of a low-efficiency promoter, causing a high percentage of false-negative cells during FACS analysis. A possible resolution in this case could be to change this construct in future experiments.

4.2. ETS1 AND FLI1 TRANSCRIPTION FACTORS ARE INVOLVED IN GC REACTIONS

To further understand the potential mechanism of action of ETS1 and FLI1, I successfully performed GEP analysis for OCI-LY7 after individual ETS1 and FLI1 down-regulation using both shRNAs against each genes and the shRNA against GFP as our control. In order to see possible differences, the same analysis was also performed with SUDHL-4 and SUDHL-2 that lack 11q24.3 gain and, as said before, well represent the two different DLBCL subtypes (GCB- and ABC-DLBCL respectively) (Fig. 41.).

GEP data were first extracted using Illumina GenomeStudio software and then analyzed with Partek program. Using this tool, I was able to detect transcripts with differences in expression. I looked for individual genes significantly affected by silencing the two ETS factors and also for the more general effect on regulatory pathways using Gene Set Enrichment Analysis (GSEA), a software that allow the biological interpretation of GEP data. This program is based on online available gene sets that are groups of genes (or pathways) that share common biological function, chromosomal location, or regulation. I determined the transcripts significantly affected by the individual shRNA (fold change ≥1.2, FDR <0.20), and then I considered only those commonly regulated by both shRNA pairs for ETS1 and by both shRNA pairs for FLI1. In OCI-LY7 ETS1 affected 331 transcripts (150 up- and 181 down- regulated) and FLI1 affected 213 transcripts (111 up- and 102 down- regulated) (see Appendix, tables 1-2 or online Supplementary tables S2-S3). Both visual inspection of the gene lists as well as the functional analysis of the changes affecting the transcriptome in its integrity highlighted that the genes affected by ETS1 and/or FLI1 silencing in OCI-LY7 code for proteins involved in cell cycle regulation, BCR signaling, PC differentiation, and chemotaxis (see Appendix, tables 7-8 or online Supplementary tables S8-S9) such as CXCR5, NFKB1 (ETS1-regulated), CXCR4, CXCR7, CDC25B, and WEE1 (FLI1-regulated). In figure 42. I show the qRT-PCR of the most important down-regulated genes after ETS1 and FLI1 silencing in OCI-LY7 that validates the results from GEP analysis.
Also in SUDHL-4 and SUDHL-2 ETS1 and FLI1 silencing affected the transcription program. More in detail, ETS1 silencing affected 1056 transcripts in SUDHL-4 and 400 transcripts in SUDHL-2, while FLI1 silencing affected 3051 transcripts in SUDHL-4 and 1040 transcripts in SUDHL-2 (see Appendix, tables 3-4-5-6 or online Supplementary tables S4-S5-S6-S7). In contrast to OCI-LY7, in these cell lines lacking the 11q24.3, ETS1 and FLI1 silencing did not appear to affect genes involved in B cell maturation pathways but only affect gene involved in pathways regulating cell proliferation, confirming the phenotypic data obtained for these cell lines (see Appendix, tables 9-10-11-12 or online Supplementary tables S10-S11-S12-S13).

Figure 41: Hierarchical clustering of samples analyzed with GEP. GEP analysis was performed after shRNA experiments targeting ETS1 (left panel) or FLI1 (right panel) in three cell lines (OCI-Ly7, left; SU-DHL4, middle; SU-DHL2, right).
4.3. ETS1 DIRECTLY REGULATES CHEMOKINE RECEPTOR CXCR5

A very interesting and new finding emerging from these data is the regulation of chemokines receptors by ETS1 and FLI1 factors. These data have never been described before and strongly reinforce the important role of ETS1 and FLI1 in GC reactions. The exact mechanisms involving chemokine receptors in GC formation and development are still unclear. Moreover, chemokine receptors are also involved in the metastasis of different kind of solid tumors. B cell homing is an important process for GC formation and development and is regulated by chemokines receptors expressed on B cells surface and chemokines expressed by follicular cells. Disruption of this process could lead to lymphomagenesis. In this context, the identification of ETS1 and FLI1 as direct regulators of chemokine receptors could fill important gaps in the knowledge of DLBCL pathogenesis. So far I focused my attention on the regulation of CXCR5 by ETS1. I was interested in understanding if ETS1 regulates CXCR5 in a direct or in an indirect way. CXCR5 promoter contains different EBSs and some of these were specific for ETS1 protein and close to the transcription start site of CXCR5 (about 250bp downstream to the ATG). Thus, I performed a ChIP experiment in which ETS1 protein-DNA complexes were isolated using an antibody against ETS1 protein. ChIP analysis was performed on two DLBCL cell lines, OCI-LY7 and SUDHL-2, without any previous treatment. Through PCR and qRT-PCR it was observed that ETS1 directly binds to CXCR5 promoter in OCI-LY7 and very weakly in
Results

SUDHL-2 (Fig. 43). PCR and qRT-PCR were performed with a pair of primers specific for flanking sequences of one of the putative EBSs inside CXCR5 promoter.

<table>
<thead>
<tr>
<th></th>
<th>UNB</th>
<th></th>
<th>EB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LY7</td>
<td>M</td>
<td>W</td>
<td>IgG</td>
<td>ETS1</td>
</tr>
<tr>
<td>SUDHL-2</td>
<td>IgG</td>
<td>ETS1</td>
<td>IgG</td>
<td>ETS1</td>
</tr>
</tbody>
</table>

Figure 43: ChIP analysis of CXCR5 promoter performed with antibody against ETS1 in OCI-LY7 and SUDHL-2 not previously treated. Upper panel: Input (UNB) and immunoprecipitated DNA (EB) samples were amplified by qRT-PCR analysis using primers specific for one EBS promoter region of CXCR5 gene. IgG samples were used as control. M, marker; W, water; IgG, sample isolated with anti-IgG antibodies; ETS1, samples isolated with anti-ETS1 antibodies. Lower panel: qRT-PCR analysis on same samples analyzed with PCR. anti-ETS1 1e and anti-ETS1 2e correspond to first ETS1-eluate and second ETS1-eluate respectively.
5. A NEW DRUG BLOCKING EWS-FLI1 ONCOGENIC PROTEIN HAS CYTOTOXIC EFFECTS AGAINST DLBCL CELL LINES

It was demonstrated that FLI1 is a pro-survival factors which down-regulation leads to high mortality in DLBCL cell lines (Figs. 30-34). In order to evaluate FLI1 as possible therapeutic target, a series of DLBCL cell lines and solid tumors were treated with a new drug, YK-4-279 previously reported blocking the transcriptional activity of the chimeric protein EWS-FLI1 in Ewing’s sarcoma. YK-4-279 is a small molecule protein–protein interaction inhibitors against EWS-FLI1 and RNA helicase A (RHA), a functional crucial partner of EWS-FLI1 fusion protein. YK-4-279 was also demonstrated to inhibit ERG and ETV1 dependent transcriptional activity leading to reduced cell motility and invasion. ERG and ETV1 are rearranged in 40-70% of prostate cancer tumors representing therapeutic targets for the treatment of this disease. FLI1, ERG and ETV1 share high identity and homology in their amino acid sequences belonging to the same subfamily of ETS factors. For this reason I tested the effects of YK-4-279 on DLBCL cell lines.

<table>
<thead>
<tr>
<th>cell lines</th>
<th>IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>0.57</td>
</tr>
<tr>
<td>SUDHL2</td>
<td>0.62</td>
</tr>
<tr>
<td>OCI-LY7</td>
<td>0.68</td>
</tr>
<tr>
<td>K422</td>
<td>0.74</td>
</tr>
<tr>
<td>HEK293T</td>
<td>0.85</td>
</tr>
<tr>
<td>SUDHL4</td>
<td>1.64</td>
</tr>
<tr>
<td>DU-145</td>
<td>3.57</td>
</tr>
<tr>
<td>MCF7</td>
<td>IC_{50}&gt;50µM</td>
</tr>
</tbody>
</table>

Figure 44: left panel: DLBCL cell lines and solid tumor-derived cell lines were treated with a dose range of YK-4-279. Cell growth, as measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenil-3H-tetrazolium bromide (MTT) after 72 hours in culture. Right panel: IC_{50} values in ascending order for each cell lines analyzed.
Results

Four different DLBCL cell line were chosen for cellular cytotoxic assay (MTT assay) (Fig. 44). Among DLBCL cell lines, OCI-LY7 cells were chosen because were the cellular model used for the functional characterization of the 11q24.3 gain in which FLI1 gene is located. Prostate cancer cell line (PC3 and DU-145), MCF7 breast cancer cell line and HEK-293T packaging cells were chosen as IC50 control of already published data. Cells were exposed to increasing concentrations of YK-4-279 (dose range: 0.15-50 μM) and the MTT assay was performed after 72 hours to determine the effects of YK-4-279 in these cell lines.

I found that YK-4-279 has an inhibitory concentration (IC50) around 1 μM for the growth of all cells analyzed. MCF7, as expected, did not die within the dose range used. This is a very preliminary data that shows the efficacy of YK-4-279 in inhibiting the growth of DLBCL cell lines and confirming FLI1 as an interesting drug target to be analyzed.

For future experiments could be interesting to understand if FLI1 target genes revealed by GEP analysis are down-regulated also after YK-4-279 treatment in OCI-LY7 and if these molecular changes are associated with phenotypic effects obtained after lentiviral infection in DLBCL cell lines.

These experiments could confirm FLI1 as an interesting drug target to be analyzed.
SECONDARY AIM

Part of the data reported in the following section has been published as: Testoni M* et al. Gains of MYC locus and outcome in patients with diffuse large B-cell lymphoma treated with R-CHOP. British Journal of Haematology 2011 Oct;155(2): 274-277.

1. GAINS OF MYC LOCUS ARE NOT ASSOCIATED WITH POOR OUTCOME IN DLBCL PATIENTS

With the aim of assessing the prognostic role of MYC gains in the R-CHOP era, I have investigated the MYC status in the series of 166 DLBCL patients treated with R-CHOP (Fig. 15 upper panel) [177, 186].

Gains affecting the MYC locus were detected in 17/166 (10%) patients, a percentage comparable to MYC translocations. No patient had more than four copies of the gene. Cases with MYC locus gain expressed MYC mRNA at high levels, as evaluated by GEP with the Affymetrix U133 plus 2.0 arrays, although not significantly more than cases without MYC gain (Fig. 45).

Gain at the MYC locus was never the only aberration and was statistically associated with the presence of other aberrations, such as gains at 13q31.3, 7p/7q, 1q, 9q, 2p16-p15, 12p/12q, 5p/5q and losses at 8p and 17p (Table 3).

Figure 45: Levels of MYC expression in patients without and with MYC gain, as evaluated with Affymetrix U133 plus 2.0.
Table 3: Association between presence of MYC locus gain and other concomitant genomic lesions among 166 cases of DLBCL as evaluated by applying Fisher’s exact test (P) followed by multiple test correction (Q).

After a median follow-up of 4.9 years (25th–75th percentiles ranging from 4 to 7 years), cases with MYC gain were associated with a poor OS (P = 0.0012) and progression-free survival (PFS) (P = 0.0353) only in the presence of concomitant del(8p) (Fig. 46). Otherwise, no significant differences in outcome were observed (Fig. 46). These data showed that the simple detection of extra copies of MYC gene did not determine a poor outcome in DLBCL patients treated with R-CHOP, contrary to translocation of MYC gene.

Figure 46: Kaplan-Meier estimates of OS according to the presence or absence of MYC gain with or without concomitant del(8p).
Discussion
DISCUSSION OF THE PRIMARY AIM OF THE THESIS

Diffuse large B cell lymphoma (DLBCL) is the most frequent non-Hodgkin lymphoma (NHL) accounting for roughly 35-40% of all cases. The diversity in clinical presentation, as well as the pathologic and biologic heterogeneity, indicates that DLBCL comprises several disease entities. It is important to better characterize DLBCL in a way to offer each patient or group of patients the most appropriate therapeutic options on the basis of molecular and genomic characteristics. Several types of therapeutic approaches have been used, among which the mAb anti-CD20 (Rituximab), but a large percentage of DLBCL patients are not cured yet. By integrating genome-wide gene expression and copy number data sets, different research groups succeeded in the characterization of at least three distinct molecular subgroups of DLBCL, as described in this thesis introduction [14, 161]. The distinct DLBCL subtypes arise from B cells at different stages of differentiation, are characterized by distinct oncogenic pathways and chromosomal aberrations and have significantly different OS (60% 5-year survival for GCB and 35% 5-year survival for ABC) following immunochemotherapy.

Previously, my research group had reported genomic profile analysis by high-density genome-wide SNP-based array (Affymetrix) performed on 166 DLBCL patients treated with anthracycline-based combination polichemotherapy with addition of the Rituximab (R-CHOP) [186]. Several MCR, defined as the minimal part of chromosomes commonly affected by alterations in a percentage of patients, were identified. Several aberrations observed were already reported in literature [161, 209] reinforcing this analysis. The relevance of genomic profile analysis is to discover aberrations involving important genomic loci that could contribute to lymphomagenesis. Interestingly, important B cell genes (such as BCL6, PAX5, PRDM1 or XBP1) are mapped in chromosome regions affected by recurrent gains and losses, and considering that GCs B cell differentiation requires a complex transcriptional program that includes both positive and negative regulators, it is not surprising that alterations of genes regulating this process have an important role in the DLBCL disease.

In this thesis, I focused my attention on the less characterized gain affecting 11q24.3 genomic locus, identified in 23% of DLBCL cases. Analysis of genes mapped into this locus revealed the presence of a set of genes not normally expressed in B cells (KCNJ1, KCNJ5, P53AIP1, and RICS), and of two genes, ETS1 and FLI1, that were already described to be involved in the normal B cell differentiation process [147, 155]. In this
Discussion

A cohort of DLBCL patients ETS1 and FLI1 were expressed with levels significantly higher in cases with the gain in comparison with those without, suggesting that the gain does lead to a deregulation of the expression of both ETS factors and that ETS1 and FLI1 could be the oncogenic targets of 11q24.3 gain. These two transcription factors are ETS family members, which represent a family of transcription factors that bind via a winged helix-turn-helix motif to specific DNA sequences, known as ETS Binding Site (EBS), on the promoter of their target genes.

ETS1 considered an oncogene involved in various biological and tumorigenetic processes, such as tumor invasion and transformation and malignant angiogenesis [113, 114]. ETS1 over-expression is observed in a variety of human tumors, like ovarian, prostate, breast and lung cancers and has a role in T cell differentiation.

High levels of ETS1 expression are also described in lymphoid organs and its down-regulation precedes B cell differentiation into PC [155]. Very recently, ETS1 has also been proposed as a master transcription factor in B cells, since it binds super-enhancers regulating the expression of numerous relevant genes [345]. Purified splenic B cells isolated from ETS1-deficient mice undergo increased terminal differentiation into IgM-secreting PCs when cultured in vitro in the presence of a synthetic TLR9 ligand (CpG ODN) [132]. Mice with a targeted mutation in the ETS1 gene (deletion of the Pointed domain) exhibit increased B cell terminal differentiation to IgM-secreting PCs [133]. These mice express low levels of ETS1 protein that was incapable to be functionally activated and show increase expression of activation markers on follicular B cells (CD20lo CD23hi) and very few marginal zone B cells (CD20hi CD23lo). These observations support the idea that ETS1 is a critical negative regulator of B cell terminal differentiation induced by TLR9, and that ETS1-deficient B cells have an intrinsic propensity to undergo differentiation to PCs, confirming the important role that ETS1 plays during B cell development process. In addition, recently, non-synonymous mutations of ETS1 gene [171] and copy number gains [192] have been reported in a subgroup of DLBCL, reinforcing the putative oncogenic role.

FLI1 is a transcription activator that plays an important role in the regulation of megakaryocyte development [346]. FLI1 can bind specific EBS and transcriptionally activates a number of genes including those encoding EGR1, multiple megakaryocytic genes, bCL2, mb-1, and RB1 [347, 348]. FLI1 is involved in Ewing's sarcoma (ES) in which functional domains of FLI1 protein are translocated under the control of EWS gene promoter. This fusion protein stimulates tumor transformation and deregulates expression of genes such as c-MYC and PS3. Moreover, over-expression of FLI1 protein in transgenic mice results also in the development of a lupus-like disease, including progressive
immune complex-mediated renal disease and ultimately premature death from renal failure. Hypergammaglobulinemia, splenomegaly, B cell peripheral lymphocytosis, and autoantibody production were prominent in these transgenic mice [144]. Reduced expression of FLI1 protein in MRL/lpr mice, a murine model of lupus, significantly decreased renal disease with an increment in survival compared with wild type [145].

In addition to these studies, in this thesis I reported that: 1. The immunohistochemical analyses performed on tissues from DLBCL patients show that ETS1 and FLI1 are expressed at high levels in DLBCL tumor biopsies and appear expressed in normal B cells from human tonsils with a gradient of expression decreasing from dark to light zone. 2. The GEP analysis performed on mRNA from DLBCL patients samples revealed higher mRNA levels of both factors in DLBCL samples in respect to hyperplasia samples. All these data suggest that both ETS factors are involved in B cell development and tumorigenesis.

Thus, I decided to further analyze the oncogenic function of ETS1 and FLI1 in DLBCL patients by molecular and phenotypic assays to better characterize their roles in DLBCL lymphomagenesis.

At first, I evaluated protein levels of ETS1 and FLI1 in all DLBCL cell lines available in my laboratory. This analysis revealed that these factors are expressed in almost all DLBCL cell lines. However, the OCI-LY7 cell line was chosen as the best in vitro model for two reasons: 1. This cell line expressed the highest ETS1 protein levels and good FLI1 protein levels. 2. Genomic profile analysis revealed that OCI-LY7 carries the exact gain at 11q24.3 genomic locus, whereas the others cell lines did not.

OCI-LY7 is usually considered of GCB origin but it is more likely representative of a type of DLBCL intermediate between GCB- and ABC-DLBCL as also indicated by Monti et al. [20]. Indeed, also in our hands, the cell line expressed markers typical of both GCB (BCL6, PAX5) and non-GCB DLBCL (IRF4, PRDM1, and XBP1). If we consider that the GEP data from DLBCL patients reveal higher ETS1 mRNA expression levels in ABC- than in GCB-DLBCL and that 11q24.3 gain was observed in both ABC- and GCB-DLBCL without associations with any DLBCL subgroups, it is possible to hypothesize that the lesion might be relevant in a subgroup of DLBCL patients derived from the non-GC phase, when the pathway CD40/NF-kB is already triggered after GC centroblast proliferation.

Because ETS1 and FLI1 are two oncogenes and mapped in a region affected by a gain, I evaluated the effects of the down-regulation of this two ETS factors in OCI-LY7 cellular model.

As shown in the results part of this thesis, a series of transfection in OCI-LY7 cell line using different strategies were performed without success. The difficulty in OCI-LY7
manipulation is in accordance with the literature indicating that lymphoma cell lines are considered to be resistant to lipofectamine and electroporation [349, 350]. For their role in immune system it is possible to hypothesize that an intrinsic mechanism could exist in B cell lines protecting them from external factors, not allowing or making siRNA difficult to enter or to be maintained into the cells. Only using lentivirus strategy I was able to obtain ETS1 and FLI1 knock down and the transduction efficiency was of about 70%, calculated on the basis of cell survival after puromycin selection. Also infection methods can face difficulties in correctly transducing B cell lines, due to the high toxicity of the method or plasmid dimensions that are too big (e.g. pCDH empty vector is 7544 bp), rendering difficult the integration of virus genome in the DNA of host cells.

After infection of OCI-LY7, the first observation was that FLI1 and ETS1 silencing with shRNAs was followed by a block in cell proliferation for this cell line that carries the 11q24.3 gain. Moreover, the decrease in cell proliferation was associated with a block in cell cycle and a high grade of cell mortality. This last effect was stronger for cells transduced with shRNAs against FLI1 in respect to ETS1. Interestingly, when both genes were concomitantly silenced, the percentage of mortality was higher respect the single silencing indicating that both genes are synergistic in supporting survival pathways in this DLBCL cell line. This observation is in accordance with that reported for 9q amplification in which two oncogenes, JAK2 and JMJD2C, act synergistically in DLBCL [96].

Looking the mortality of all the other DLBCL cell lines infected with the shETS1 and shFLI1, I observed that ETS1 down-regulation did not affect the survival even though it affected cell proliferation, while FLI1 silencing was always cytotoxic. Hypothetically, FLI1 was the best candidate involved in cell survival because from the literature it is known that FLI1 is involved in regulation of cell cycle and apoptosis, and able to down-regulate the expression of the retinoblastoma (Rb) protein at the transcriptional level leading to the transition of cells through the S phase [114, 137]. In addition, the survival-inducing property of FLI1 is associated with increased transcription of BCL2: in erythroblasts, FLI1 binds BCL2 promoter sequences and it is able to inhibit apoptosis in the absence of Epo [137].

The important role of FLI1 on cell proliferation and survival could represent the bases for possible new therapy specifically targeting FLI1 protein. Being FLI1 a regulator of important ubiquitous factors, such as BCL2, c-MYC and Rb, FLI1-specific drugs might potentially have toxicity. However, neoplastic cells might be more sensitive than normal cells providing a therapeutic window. Now, I have been testing a new drug, Y-4-279. This small molecule was created to inhibit EWS-FLI1 fusion protein activity in ES disease and was seen to work also against ERG1 and ETV1 protein activity in prostate cancer, leading to
the down-regulation of their specific target genes. Up to now, I cannot assert that this
drug specifically inhibits FLI1 protein activity, but also for lymphoma cell lines this drug has
phenotypic effects similar to those observed for ES cell lines.
Regarding the possible role and regulation of ETS1 and FLI1 during B cell differentiation,
different studies reported that ETS1 and FLI1 have a role in regulating immunity processes
such as impairment of T cell differentiation without ETS1 and the development of a lupus-
like disease in the absence of FLI1. Moreover, ETS1 and FLI1 were already described as
important factors during early GC B cell developmental phases regulated by Ca\(^{2+}\)-
loaded Calmodulin (CaM) factor via the activation of BCR pathway (Fig.10 and 47).
Thus, considering also previous genomic profile, immunohistochemical and GEP data on
DLBCL patients, and considering that OCI-LY7 DLBCL cell line requires ETS1 and,
especially, FLI1 expression to survive, it is possible to hypothesize that 11q24.3 gain might
be an additional tumorigenic mechanism in DLBCL that leads, via deregulating ETS1 and
FLI1 expression, to escape the GC regulatory inhibition mechanisms.
However, how do ETS1 and FLI1 carry out their oncogenic role in DLBCL patients bearing
11q24.3 gain? Which are their specific targets during B cell differentiation?
Using qRT-PCR analysis, I evaluated the possible role of ETS1 and FLI1 on the expression of
different genes involved in PC differentiation and I observed an up-regulation of PRDM1
and XBP1 after ETS1 down-regulation. This effect was already described by John SA et al
in 2008 [132] in a primary B cell model stimulated with CpG ODN to induce PC
development, in which ETS1 interfered with BLIMP1 activity. Moreover, ETS1 must be
down-regulated during the late phases of PCs differentiation, exactly when BLIMP1 is
acting in order to allow B cells development (Fig.10 and 47) [155]. Thus, I further analyzed
this possible interaction demonstrating, through a ChiP assay, that ETS1 directly bind to
PRDM1 promoter interfering with its expression.
Considering that BLIMP1 is a tumor suppressor factor, the critical role of ETS1 on cell
viability observed only for OCI-LY7 cell line could be explained as a consequence of
BLIMP1 up-regulation following ETS1 down-regulation. Indeed, SUDHL-4, bearing the
classical features of GC-derived cells, did not express PRDM1 at mRNA or protein levels
(Fig.40). ETS1 down-regulation was not followed by PRDM1 mRNA up-regulation in U2932
cell lines (Fig.40), in which the constitutively deregulated BCL6 represses the PRDM1
gene. Accordingly, SUDHL2 also carry PRDM1 inactivating mutations.
These results suggest that down-regulation of ETS1 followed by the up-regulation of
BLIMP1 may be more essential for achieving effective plasmacytic differentiation then
cell survival. This idea was reinforced by the up-regulation of XBP1 next to the up-
regulation of BLIMP1. XBP1 is required for PC formation and Ab production and its
expression is increased after PC differentiation is triggered. Contrarily, FLI1 did not regulate any tested genes important for B cell development. Actually, FLI1 normally regulates BCL2 expression, but in OCI-LY7 BCL2 is gained thus probably its expression is FLI1 independent.

Figure 47: ETS1 and FLI1 regulation during GC processes in a normal B cell. Normally, after CD40-CD40L stimulation, NF-kB pathway is activated. This results in the activation of BLIMP1 and XBP1 and in the transcriptional silencing of GC transcription factors BCL6, PAX5 and SPIB. Via BCR stimuli occurring with the binding to antigens exposed by FDC, the Ca²⁺-loaded Calmodulin (CaM) pathway is trigger leading to a down-regulation of ETS1 and FLI1. All these mechanisms are likely to be crucial for the transition of GC B cells from centroblast to centrocytes committed to PC differentiation. Black lines with stop bars indicate inhibitory regulations. Green and black arrows indicate activating regulations of specific genes or pathways. Blue arrow indicates down-regulation of the alongside gene. Red arrows indicate up-regulation of alongside genes.

To better understand which pathways are deregulated after ETS1 and FLI1 silencing and to better realize how these two transcription factors are involved in tumorigenesis, a GEP analysis was performed on OCI-LY7 DLBCL cell model and other two DLBCL cell lines lacking the gain, SUDHL-4 and SUDHL-2. To interpret gene expression data I used GSEA analytical method. As additional DLBCL cell lines, I chose SUDHL-4 and SUDHL-2 because well represent the two main DLBCL subgroups: SUDHL-4 is a GCB-like DLBCL cell line expressing BCL6 and PAX5 at high levels while does not express IRF4, BLIMP1 and XBP1. In contrast, SUDHL-2 is an ABC-like DLBCL cell line expressing IRF4 and XBP1 factors but not BCL6 and PAX5. I observed that the genetic silencing of ETS1 and FLI1 caused a
deregulation of genes coding for factors involved in cell cycle and B cell development in OCI-LY7 bearing the 11q24.3 gain, while mainly genes involved in cell cycle regulation appeared differentially expressed in the remaining analyzed DLBCL cell lines. In all the cell lines FLI1 mainly regulated cell cycle and apoptotic pathways as attended, while ETS1 deregulated B cell specific pathways such as CD40 pathway, only in OCI-LY7 cell line and not in the other cell lines. These results were in accordance with previous phenotypic and molecular data confirming that FLI1 is principally involved in cell proliferation and survival whereas ETS1 became critical when deregulated by genomic lesions not only for cell survival but also for causing impairment of the PC differentiation pathway.

An interesting finding of our study was that both ETS1 and FLI1 genes appeared to regulate the expression of chemokine receptors. ETS1 silencing caused a down-regulation of CXCR5, while FLI1 silencing caused a down-regulation of CXCR4 and CXCR7.

The two chemokine receptors regulate the development and the trafficking of B cells within the GC [83]. In normal lymphoid tissue, CXCR4 is highly expressed in the dark zone of the GC [78], attracting the centroblasts, whereas CXCR5 expression occurs in the light zone [71] and contributes to attracting B cells to the area and is then down-regulated during plasmacytic differentiation. CXCR4 signaling has also a pro-survival role in B cells [351], and, in a subset of DLBCL, FLI1 might promote B cell survival through a positive regulation of CXCR4. As a whole, importance of CXCR7 in the immune compartment is well established. B cells lacking CXCR7 show diminished accumulation in lymph nodes compared with wild-type counterparts [74, 75]. However, the clear function of CXCR7 in B cell development is not yet fully clear.

The effect of ETS1 and FLI1 on different genes involved in similar pathways was in accordance with other important observations, all indicating that the 2 ETS factors might contribute to DLBCL growth in a cooperative manner. First, the 2 genes are closely mapped, indicating that they might arise by gene duplication from a common ancestral gene and thus be coaffected by the 11q24.3 gain. Second, when both genes were down-regulated in the OCI-LY7 cell line, the observed effects were much more severe than with single gene silencing. Third, ETS1 and FLI1 RNA expressions were correlated across DLBCL samples.

Up to now, I have further analyzed the regulation of CXCR5 by ETS1. This is a new molecular mechanism, never described before in B cell models, and could have relevance for designing specific drugs that inhibit this regulatory mechanism, not only for DLBCL lymphoma but also for other kind of tumors in which both, ETS1 and CXCR5, are
involved in tumor progression, such as breast or prostate cancers. I have described a possible direct binding of ETS1 on CXCR5 promoter but several questions are still open yet.

All my results, already published on Blood journal [352], allow to design a model of the role of ETS1 and FLI1 in DLBCL patients carrying the 11q24.3 gain (Fig.48). As previously shown in figure 47, under normal conditions, signalling from BCR-Ag and CD40–CD40L interactions result in the nuclear translocation of NF-kB subunits that are fundamental determinants of early PC differentiation.

**Figure 48:** In a DLBCL patients carrying the 11q24.3 gain, ETS1 and FLI1 are constitutively expressed. Their deregulation leads to BLIMP1 down-regulation by the up-regulation of ETS1, and in an advantage in cell survival and proliferation especially thanks to FLI1 up-regulation. These effects are in addition to other mechanisms of action that might be responsible for the blocking of the entrance of B cells into PC differentiation. Blue and red lines with stop bars indicate inhibitory regulations of specific genes or B cell differentiation stages. Red arrows indicate up-regulation of alongside genes. Green and black arrows indicate activating regulations of specific genes or pathways. Trace arrows indicate hypothetical regulations of our model.
On one hand, translocation of NF-κB factor into the nucleus activates post-GC gene-regulatory network that leads to a down-regulation of GC transcription factors such as PAX5, BCL6 and SPIB. Activation of this gene-regulatory network leads to the up-regulation of BLIMP1 that is essential for the generation and for the prolonged maintenance of PCs. In the other hand, BCR signalling, leads to the down-regulation of others important GC transcriptional factors such as ETS1 and FLI1, whom this thesis try to clarify the pathogenetic role.

Conversely, during DLBCL post-GC gene-regulatory network is altered because of several aberrations affecting different B cell genes all possibly contributing to a block in PC differentiation. In particular, for a subgroup of DLBCL patients derived from the transformation of late GC B cells, the acquisition of the 11q24.3 gain causes a constitutive high expression of ETS1 and FLI1 genes and might allows both transcription factors to escape from normal PC regulatory mechanisms. Thus, ETS1 can directly inhibit BLIMP1 expression, while FLI1 can act on the survival of tumor cells. All these processes occur together with others mechanisms of action, such as regulation of chemokine receptors that contribute in maintaining GC conditions leading to a block in PC differentiation (Fig.48). Moreover, this mechanism could be a basis for new therapies. In fact, in addition to FLI1 therapeutic implications explained before, also the effect of ETS1 on PRDM1 gene could have a very important interest. PRDM1 is the master regulator of PC differentiation and, being a tumor suppressor gene, is frequently found inhibited in DLBCL patients.

Thus, drugs targeting ETS1 protein in patients carry the 11q24.3 gain or having a constitutive expression of ETS1 gene, could indirectly restore PRDM1 expression and/or activity leading to tumor cells mortality.

ETS1 and FLI1 are two transcription factors ubiquitously expressed, drug therapy against these proteins could cause high toxicity in patients. But considering that tumor cells maybe are more sensitive to drugs against ETS1 and FLI1 oncogenes, if these drugs are used in combination with other molecules, as for several new agents currently used for DLBCL therapy (chapter 4), could then represent a good alternative for the treatment of the most aggressive DLBCL cases with high tumor proliferation rate.

In conclusion, the role of 11q24.3 gain has been elucidated in this thesis. This new recurrent gain has not any association with one of the two major DLBCL subgroups and has not a clinical impact for the prognosis of a specific subgroup of DLBCL patients. However, 11q24.3 gain leads to the deregulation of two important transcription factors involved in important GC pathways and in the survival of B cells.
Hence, the toxic effects on DLBCL cell lines and the deregulation of important GC and post-GC genes after the down-regulation of ETS1 and FLI1 demonstrate that these two factors have a role in DLBCL lymphomagenesis.
DISCUSSION OF THE SECONDARY AIM OF THE THESIS

As previously discussed, DLBCL is defined as a neoplasm which has a diffuse growth pattern. DLBCL represent a potentially curable disease when treated with R-CHOP which is considered the current standard chemotherapy. Nevertheless, due to the high heterogeneity of DLBCL genome aberrations, a too high percentage of patients are not cured yet. Gene expression profiling (GEP) patterns and genomic aberrations have been identified with an impact on the clinical course of DLBCL patients treated with R-CHOP. The MYC oncogene is one of the major regulators of normal and neoplastic B cells and their chromosomal translocations occur in DLBCLs with a frequency of 5–15% [173]. The translocation t(8;14)(q24;q32) deregulates the gene by juxtaposing it to the immunoglobulin heavy chain gene promoter and it is the most typical lesion of Burkitt lymphoma. MYC can also be deregulated by chromosomal translocations in diffuse large B-cell lymphoma (DLBCL). Different studies demonstrated that MYC translocations are significantly associated with worse survival in patients with DLBCL treated with R-CHOP suggesting to include the evaluation of MYC status in the diagnostic panel for newly diagnosed DLBCL [115, 116, 120, 121, 180, 184]. On the contrary, the role of MYC copy number gain in the prognosis of DLBCL patients in the R-CHOP era is still debated. From the analysis of our cohort of DLBCL patients the extra copies of MYC gene do not affect the outcome of the patients unless concomitant to the deletion of chromosome arm 8p (Fig. 46). Importantly MYC gain was never the only aberration affecting DLBCL patients analyzed but was associated with several other genomic aberrations (Table 3). In conclusion, there are clear indications from the literature for the potential benefit of including the evaluation of MYC gene status by FISH in the diagnostic panel of newly diagnosed DLBCL patients. However, these data showed that the detection of extra copies of MYC did not determine a poor outcome in DLBCL patients treated with R-CHOP. Thus, proper attention should be paid by clinicians to the type of lesion reported, especially if patient-tailored therapeutic plans are to be considered. Prospective evaluation of MYC status in large series of cases will clarify the role of gains versus translocations in predicting a poor outcome in DLBCL patients.
Deregulation of ETS1 and FLI1 contributes to the pathogenesis of diffuse large B-cell lymphoma

Paola Bonetti, Monica Testoni, Marta Scandurra, Maurilio Ponzoni, Roberto Piva, Afua A. Mensah, Andrea Rinaldi, Ivo Kwee, Maria Grazia Tibiletti, Javeed Iqbal, Timothy C. Greiner, Wing-Chung Chan, Gianluca Gaidano, Miguel A. Piris, Franco Cavalli, Emanuele Zucca, Giorgio Inghirami and Francesco Bertoni
Diffuse large B-cell lymphoma (DLBCL) is the most common form of human lymphoma. DLBCL is a heterogeneous disease characterized by different genetic lesions. We herein report the functional characterization of a recurrent gain mapping on chromosome 11q24.3, found in 23% of 166 DLBCL cases analyzed. The transcription factors ETS1 and FLI1, located within the 11q24.3 region, had significantly higher expression in clinical samples carrying the gain. Functional studies on cell lines showed that ETS1 and FLI1 cooperate in sustaining DLBCL proliferation and viability and regulate genes involved in germinal center differentiation. Taken together, these data identify the 11q24.3 gain as a recurrent lesion in DLBCL leading to ETS1 and FLI1 deregulated expression, which can contribute to the pathogenesis of this disease. (Blood. 2013;122(13):2233-2241)
the master regulator of plasma cell differentiation, PRDM1. Notably, Ets1 and Flil are repressed during the late phases of B-cell differentiation in an in vitro murine system. Here, we show that the 11q24.3 genomic lesion correlates with high levels of ETS1 and FLI1 expression in human DLBCL primary samples. Moreover, we demonstrated, in an in vitro model, that both ETS1 and FLI1 are critical for cell viability and that they regulate genes that are controlling the normal GC B-cell differentiation.

Methods

Tumor panel

DNA profiles, obtained using the GeneChip Human Mapping 250K NspI (Affymetrix, Santa Clara, CA), of 166 DLBCL samples from a previously published series were investigated (GSE15127). Genomic profiles of DLBCL cell lines were obtained using the 250K or single nucleotide polymorphism 6.0 array, as previously described. Gain was validated by fluorescence in situ hybridization using FLI1/EWSR1 probe (CytoCell, Cambridge, UK). Gene expression data of DLBCL clinical specimens were obtained using the Affymetrix Genechip U133 plus 2.0 (Affymetrix) (GSE10846). CEL files were imported and normalized using robust multivariate average algorithm in Partek Genomics Suite 6.4 (Partek, St. Louis, MO). Box-plots were created with Stata/SE v.12.1 (StataCorp, College Station, TX), and differences of expression between cases with and without the 11q24.3 gain were evaluated with a 1-sided test for unequal data with unequal variance. The distinction of DLBCL cases in GCB or in ABC was available in 134 cases: assessed in 77 cases by immunohistochemistry according to the algorithm of Hans et al (28 GCB, 47 non-GCB) and in 57 by GEP (30 GCB, 27 non-GCB/ABC), as previously described. All patients provided informed consent in accordance with the hospital’s institutional review board and the Declaration of Helsinki.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 4-μm-thick sections from 12 cases of DLBCL underwent immunohistochemical characterization with antibodies against ETS1 (1G11, Novoceastra, Nunningen, Switzerland) and FLI1 (G146-222, BD Pharmingen, Bucinnasio, Italy).

Short hairpin RNAs and plasmid constructs

The short hairpin RNA (shRNA) molecules were obtained from the Expression Arrest TRC library (Sigma-Aldrich, St. Louis, MO). Human pLKO.1 lentiviral shETS1 used were TRCN0000005588 and TRCN0000005591, or shFLI1 (TRCN0000005324 and TRCN0000005326). pLKO.1 GFP expressing vector was constructed as previously described. ETS1 expressing vector was cloned in pCMV6-Entry vector (Origene, Rockville, MD).

Cell culture and lentiviral infection

HEK293T cells were cultured under standard conditions (37°C in humidified atmosphere, with 5% CO₂) in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (FCS). DLBCL cell lines (OCI-Ly7, SU-DHL4, Val, U-2932, SU-DHL2, and OCI-Ly10) were obtained from Dalla Favera and Staudt laboratories. OCI-Ly7, SU-DHL4, Val, and U-2932 were maintained in RPMI1640 supplemented with 10% FCS and 1% penicillin/streptomycin and 2 mM glutamine. OCI-Ly7 medium was supplemented with nonessential amino acids (100 μM), Na-pyruvate (1 mM), and β-mercaptoethanol (50 μM). SU-DHL2 and OCI-Ly10 were maintained in Iscove modified Dulbecco medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM glutamine. Transfections of HEK-293T were performed with JetPrime reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer’s instructions. HEK293T were transfected with pLKO.1 lentiviral vector in combination with third-generation helper plasmids. Self-inactivating lentiviral particles were produced as previously described. Exponentially growing cells (3 x 10⁶/mL) were infected with viruses and puromycin added to the medium 48 hours after infection. Alive cells were recovered after 72 hours and collected at different days.

Electroporation

OCI-Ly7 cells were transiently transfected with Amazax Nucleofector methodology (Amazax Inc., Gaithersburg, MD). Briefly, 5 x 10⁶ cells per nucleofection sample were resuspended in 100 μL of nucleofector solution V. Two micrograms of pCMV6-ETSI or control pCMV6-EV plasmid DNA was added to each cell suspension and transferred to an Amazax-certified cuvette. Nucleofection was performed using the O-17 program.

Real-time polymerase chain reaction (PCR)

RNA was extracted using the RNA easy kit (Qiagen AG, Hombrechtikon, Switzerland) and reverse-transcribed using the Superscript First-Strand Synthesis System for real-time PCR kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions.

Western blotting analysis

Cells were solubilized in hot SDS lysis buffer (2.5% SDS, Tris-HCl pH 7.4) and sonicated for 15 s. The following primary antibodies were used: anti-ETS1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLI1 (C-19, Santa Cruz Biotechnology), anti-PRDM1 (6D3, Santa Cruz Biotechnology) and anti-GAPDH (MAB374, Millipore, Zug, Switzerland), anti-BRF4 (no. 4964, Cell Signaling technology), and anti-XBP1s (clone 143F, Biologic, CNIO, Spain).

Proliferation assays

Cells infected with pLKO.1shGFP, shETS1, or shFLI1 were seeded in triplicate in 24-well plates after puromycin selection and counted daily for 5 days. For EdU labeling, 10⁶ OCI-Ly7 cells were pulsed with EdU (10 μM) for 2 hours at 37°C in 5% CO₂. EdU incorporation was determined by flow cytometry using the Click-iT EdU Alexa488 Kit (Invitrogen) according to the manufacturer’s instructions.

For competition assay, OCI-Ly7 cells infected with pLKO.1shGFP, shETS1, or shFLI1 (GFP negative) were mixed with an equal number of OCI-Ly7 cells infected with pLKO.1 GFP-expressing vector (GFP positive) and the percentage of GFP positive cells was evaluated by fluorescence-activated cell sorter analysis at days 3, 7, 10, 17, and 24.

Analysis of apoptosis by flow cytometry

Apoptosis was measured by flow cytometry after staining with annexin V and propidium iodide. Annexin V binding was revealed by incubation with FITC. Cells were analyzed by FACScan using CellQuest Program.

Chromatin immunoprecipitation

OCI-Ly7 cells infected with shGFP and shETS1 were collected, cross-linked with formaldehyde, and processed as previously described. After sonication, chromatin was immunoprecipitated with antibody for ETS1 (C-20 X, Santa Cruz Biotechnology). DNA-protein cross-links were reversed and DNA purified from total cell lysates (input) and immunoprecipitated fractions. Quantitative real-time PCR was performed using SYBR Green qPCR and the following primers: PRDM1 FW 5’-GAGAAGCAGGAATGTCGGAGG-3’ and PRDM1 REV 5’-AGCGTCCAGGCGACTTAAT-3’. The amount of immunoprecipitated DNA was calculated in reference to a standard curve and normalized to input DNA.
Gene expression profiling was performed after single ETS1 and FLI1 down-regulation using 2 shRNAs against each gene in 2 independent experiments. Total RNA was isolated using Trizol (Invitrogen Life Technologies). The concentration and the quality of total RNA were assessed as previously performed. 28 Samples were processed using the HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA), according to the manufacturer’s protocol. Arrays were read on an Illumina HiScanSQ system. Data were first extracted with the Illumina GenomeStudio software and then imported in Partek Genomics Suite 6.4 and quantile normalized. Transcripts with differences in expression were identified by ANOVA using both shRNA for each gene against shGFP. Genes were considered significant for absolute fold change >1.2 and false discovery rate <0.20. Functional annotation was performed using the Gene Set Enrichment Analysis, 29 and the enrichment was calculated for “chemical and genetic perturbations” and “canonical pathways” gene sets. Gene Set Enrichment Analysis default settings were used to define enrichment of gene sets. Raw data will be available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database.

Results

A recurrent 11q24.3 gain is associated with overexpression of ETS1 and FLI1

The analysis of the genomic profiles of 166 cases of DLBCL by high-density genome-wide single nucleotide polymorphism-based array 21 had originally identified a minimal recurrent region of gain, not previously functionally characterized, mapping at 11q24.3 (GRCh37/hg19chr: 128 137,400-128 994,334) in 23% (38/166) of the cases. The region contained 6 genes: ETS1, FLI1, KCNJ1, KCNJ5, TP53AIP1, and ARHGAP32. Integration of genomic profiles with gene expression profiles in a set of 54 cases (16 bearing 11q24.3 gain) showed that, among these 6 genes, only the ETS1 and FLI1 genes were expressed (Figure 1A). Cases with 11q24.3 gain presented significantly higher RNA levels of ETS1 (fold change: 1.4, P = .0129) and FLI1 (fold change: 1.5, P = .0082) than wild-type cases.
As assessed on the GSE10846 dataset was no significant difference between the 2 DLBCL subtypes or in respect to the benign hyperplasia. There were higher ETS1 and FLI1 levels in ABC and GCB DLBCL (Figure 1A). There was also a positive correlation between the 2 mRNA levels across all DLBCL cases (correlation coefficient 0.73; Figure 1B). Expression was also confirmed at the protein level on 12 DLBCL cases, 4 bearing the gain (supplemental Figure 1). There was no significant association between 11q24.3 gain and the DLBCL cell of origin. As assessed on the GSE10846 dataset (167 ABC DLBCL, 183 GCB DLBCL, 66 unclassified DLBCL), ETS1 levels were higher in ABC and GCB DLBCL cases than in 9 samples of benign hyperplasia (P = .013 and P = .035, respectively) and in ABC than in GCB DLBCL (P < .001). FLI1 expression levels did not show any difference between the 2 DLBCL subtypes or in respect to the benign hyperplasia.

**ETS1 and FLI1 sustain cell viability of a DLBCL cell line**

To study a putative pathogenetic role of ETS1 and FLI1 in DLBCL, we first screened 22 DLBCL cell lines, encompassing GCB and ABC subtypes, for ETS1 and FLI1 protein levels and found that most of them expressed ETS1 and FLI1, albeit at different levels. By combining these data with genome-wide DNA profiling, we found that among the evaluated cell lines, the OCI-Ly7 DLBCL cell line presented both the 11q24.3 gain observed in clinical specimens and the highest protein levels of ETS1 and FLI1 (Figure 2A; supplemental Figure 2). Thus, we considered the OCI-Ly7 DLBCL cell line a suitable model to in vitro study the pathogenetic roles of ETS1 and FLI1 in DLBCL.

We next down-regulated the ETS1 and FLI1 expression in OCI-Ly7 cells through lentiviral shRNA, utilizing 2 different hairpins for each gene (nos. 1 and 2). Both real-time PCR and western blotting analyses revealed significant reductions of the levels of the 2 ETS factors with respect to the shGFP control vector (Figure 2B). We then examined the effects of ETS1 and FLI1 down-regulation on cell proliferation and viability in the OCI-Ly7 cell line. We found that both ETS1 and FLI1 down-regulation induced a lower proliferation rate compared with the shGFP control (Figure 2C), and this phenotype was associated with a reduced percentage of cells in S-phase (Figure 2D). To evaluate the biological effects of ETS1 and FLI1 down-regulation at longer time points, we added cells infected with shRNA against ETS1 or FLI1 (GFP negative) to an equal amount of cells infected with a vector carrying the GFP marker (GFP positive). Fluorescence-activated cell sorter analysis of the percentage of the 2 cell populations at days 3, 7, 10, 17, and 24 following infection demonstrated that OCI-Ly7 cells with reduced levels of ETS1 or FLI1 proliferated less efficiently and were outgrown by GFP positive cells, at a variance with cells infected with the control vector (shGFP), thereby indicating an impairment of cell proliferation as a result of the ETS1 or FLI1 down-regulation (Figure 2E). Control shRNA transduced cells did not display this readout. In comparison with shETS1 cells, shFLI1 cells were more rapidly overcome by GFP-positive cells despite a similar percentage of regulation for both proteins, suggesting a more prominent role for FLI1 in regulating cell proliferation. We then investigated whether this phenotype was associated with cell death and found that down-regulation of ETS1 or FLI1 induced apoptosis in a significant percentage of OCI-Ly7, indicating that these 2 genes are critical for cell viability in this DLBCL cell line (Figure 2F).

To investigate whether ETS1 and FLI1 in 11q24.3 gain positive patients could cooperate in regulating cell viability; we concomitantly down-regulated both genes in OCI-Ly7 (Figure 3A). Concomitant down-regulation of ETS1 and FLI1 clearly induced apoptosis in a percentage of cells that was significantly higher (70%) than those observed after individual down-regulation of ETS1 or FLI1. This strongly indicated that ETS1 and FLI1 can cooperatively sustain cell viability in DLBCL (Figure 3B).
ETS1 and FLI1 affect genes involved in B-cell terminal differentiation

Because ETS factors are involved in B-cell differentiation and in the regulation of important players in GC differentiation, we then evaluated whether ETS1 and FLI1 regulate the expression of known genes playing a key role in GC B-cell development in the OCI-Ly7 DLBCL cell line in order to elucidate the mechanisms through which ETS1 and FLI1 might exert their role in DLBCL. We analyzed the mRNA levels of BCL2, BCL6, PAX5, IRF4, PRDM1, and XBP1 following knocking-down of ETS1 and FLI1. ETS1 down-regulation resulted in a significant up-regulation of PRDM1 and this effect was maintained, even when both ETS factors were down-regulated (Figure 4A-B). Similarly, silencing of ETS1 determined a XBP1 up-regulation, whereas no effect on IRF4 was observed, suggesting that ETS1 might act up-stream or independently from the NF-κB pathway (Figure 4A-B). We then reinduced ETS1 expression in OCI-Ly7 cells previously infected with shETS1 and observed that ETS1 reintroduction restored PRDM1 mRNA levels to values similar to the control cells (shGFP) (Figure 4C), indicating that the observed PRDM1 up-regulation was induced by ETS1 silencing. In order to evaluate whether PRDM1 was directly regulated by ETS1, we looked for the presence of ETS binding site (EBS) on PRDM1 promoter and found a canonical EBS (−1035 bp). We performed chromatin immunoprecipitation by using antibody against ETS1 on OCI-Ly7 infected
with the control shGFP vector and with shETS1. We detected an enrichment of ETS1 in the PRDM1 promoter region containing the EBS in OCI-Ly7 cells that was reduced when ETS1 expression was down-regulated (Figure 4D). We can thus conclude that ETS1 negatively regulates PRDM1 transcription.

To further understand the potential mechanism of action of ETS1 and FLI1 in our DLBCL model, we performed gene expression profiling in OCI-Ly7 after individual ETS1 and FLI1 down-regulation using 2 shRNAs against each gene in 2 independent experiments (supplemental Figure 3). We looked for the individual genes significantly affected by silencing the 2 ETS factors and also for the more general effect on regulatory pathways. We determined the transcripts significantly affected by the individual shRNA (fold change >1.2, false discovery rate <0.20), and then we considered only those commonly regulated by both shRNA pairs for ETS1 and by both shRNA pairs for FLI1. ETS1 affected 331 transcripts (150 up- and 181 down-regulated) and FLI1 affected 213 transcripts (111 up- and 102 down-regulated) (supplemental Tables 2-3). Both visual inspection of the gene lists as well as the functional analysis of the changes affecting the transcriptome in its integrity highlighted that the genes affected by ETS1 and/or FLI1 silencing code for proteins involved in cell cycle regulation, BCR signaling, plasma cell differentiation, and chemotaxis (supplemental Tables 8-9), such as CXCR5, NFKB1 (ETS1-regulated), CXCR4, CXCR7, CDC25B, and WEE1 (FLI1-regulated) (Figure 5).

Only FLI1 is essential for cell viability of DLBCL cells without 11q24.3 gain

To assess whether ETS1 and FLI1 are required for cell viability in general in primary DLBCL independently on the presence of the 11q24.3 gain and high levels of ETS1 and FLI1 expression, we down-regulated the expression of these 2 molecules in a set of GCB (SU-DHL4, Val) and ABC (OCI-Ly10, SU-DHL2, and U-2932) DLBCL cell lines lacking the 11q24.3 gain (Figure 6A). Only FLI1 down-regulation significantly affected cell viability in a way similar to what observed in OCI-Ly7 cell line. Conversely, ETS1 down-regulation did not induce apoptosis (Figure 6B) although it did decrease cell proliferation (Figure 6C). These data suggested a different role of the 2 genes in DLBCL models (Figure 6B). Notably, in the majority of the DLBCL cell lines, ETS1 down-regulation induced a significant FLI1 up-regulation in line with previous reports in normal T cells, suggesting that elevated FLI1 levels might compensate for ETS1 down-regulation. To further investigate the molecular effects of the down-regulation of ETS1 and FLI1 in DLBCL cell lines without 11q24.3 gain, we performed the same GEP experiment performed in the OCI-Ly7 cell line on 2 other DLBCL models, SU-DHL4 and SU-DHL2, and GCB- and ABC- models, respectively (supplemental Figure 3). Also in these cell lines, ETS1 and FLI1 silencing affected the transcription program. ETS1 silencing affected 1056 transcripts in SU-DHL4 (supplemental Table 4) and 400 transcripts in SU-DHL2 (supplemental Table 5), while FLI1 silencing affected 3051 transcripts in SU-DHL4 (supplemental Table 6) and 1040 transcripts in SU-DHL2 (supplemental Table 7). In these cell lines lacking the 11q24.3, ETS1 and FLI1 silencing did not appear to affect genes involved in B-cell maturation pathways (only on pathways regulating cell proliferation), confirming the phenotypic data obtained in these cells (supplemental Tables 10-13).

Discussion

In our genome-wide DNA profiling study, 23% of DLBCL samples carried a recurrent gain on chromosome 11q24.3. Among the 6 genes encompassed in the minimal common region, only the genes coding for the transcription factors ETS1 and FLI1 resulted to be expressed, with levels significantly higher in cases with the gain in comparison with those without. Functional studies on an in vitro model of DLBCL carrying the 11q24.3 gain and expressing high levels of ETS1 and FLI1 showed that these transcription factors cooperate in sustaining neoplastic B-cell proliferation and viability and affect the regulation of genes involved in the maturation process from GC B cell to plasma cell. ETS1 and FLI1 are 2 ETS family members known to be involved in tumorigenesis and B-cell development. ETS1 overexpression is observed in a variety of human tumors. Recently, nonsynonymous mutations of ETS1 gene and copy number gains have been

Figure 5. ETS1 and FLI1 affect genes involved in GC reaction. (A) Real-time PCR validation of down-regulation of CXCR5 and NFKB1 observed by gene expression profiling after ETS1 silencing. Histogram graphs represent the average of 4 independent silencing experiments. All down-regulation had a P value <0.05. (B) Real-time PCR validation of down-regulation of CXCR4, CXCR7, CDC25B, and WEE1 observed by gene expression profiling after FLI1 silencing. Histogram graphs represent the average of 4 independent silencing experiments. All down-regulation had a P value <0.05.
and contributes to the control of mice, FLI1 regulates B-cell differentiation, proliferation, and apoptosis. Ly10 also carry PRDM1 inactivating mutations. In the regulation of genes coding for proteins involved in cell cycle and B-cell development in the cell line bearing the 11q23 gain, while mainly genes involved in cell cycle regulation appeared differentially expressed in the remaining analyzed DLBCL cell lines. These results suggest that ETS1-mediated suppression of PRDM1 expression, as observed in the presence of 11q24.3 gain, would act as an alternative mechanism contributing to the pathogenesis of a subgroup of DLBCL in which constitutively high levels of ETS1 and FLI1 would deregulate the GC/plasma cell expression program.

In conclusion, the combination of genomic and expression profiling suggested a new pathogenetic mechanism for a subgroup of DLBCL derived from the transformation of late GC B cells. A model could be proposed in which the acquisition of the 11q24.3 gain causes a constitutive high expression of ETS1 and FLI1 genes.
that would deregulate genes involved in the GC expression program and on cell proliferation.

Acknowledgments

This work was supported by the San Salvatore Foundation ( Lugano, Switzerland), Oncosuisse OCS-1939-8-2006 (Bern, Switzerland), Nelia et Amadeo Barletta Foundation (Lausanne, Switzerland), and Fondazione per la Ricerca e la Cura sui Linfomi in Ticino (Bellinzona, Switzerland). M.T. and M.S. are enrolled in the program in Pharmaceutical Sciences, University of Geneva, Switzerland.

Authorship

Contribution: P.B. designed and performed experiments; interpreted data, and co-wrote the manuscript; M.T. performed experiments, interpreted data, and helped write the manuscript; M.S. performed experiments and genomic profiling; M.P. performed immunohistochemistry, provided advice, and collected and characterized DLBCL samples; R.P. provided advice; A.A.M. performed experiments and provided advice; M.G.T. performed fluorescence in situ hybridization analyses; A.R. performed genomic and gene expression profiling; I.K. performed statistical analysis; J.I. interpreted gene expression profiling data; T.C.G., W.-C.C., G.G. M.A.P., F.C., and E.Z. provided advice and collected and characterized DLBCL samples; G.I. provided advice and collected characterized DLBCL samples; and F.B. designed the study, interpreted data, and co-wrote the manuscript. All authors have approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for P.B. is Italian Institute of Technology, Center for Genomic Sciences, Milan, Italy.

Correspondence: Francesco Bertoni, Lymphoma and Genomics Research Program, IOR Institute of Oncology Research, via Vincenzo Vela 6, 6500 Bellinzona, Switzerland; e-mail: frbertoni@mac.com.

References


Correspondence

Sally Killick ²
Paresh Vyas ³
Eva Hellström-Lindberg ⁴
James S. Wainscoat ¹
Jacqueline Boultwood ¹

¹LLR Molecular Haematology Unit, NDCLS, John Radcliffe Hospital, Oxford, UK, ²Department of Haematology Oncology, University of Pavia Medical School, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, ³Medizinische Klinik II, St Johannes Hospital, Dusseldorf, Germany, ⁴Department of Medicine, Division of Haematology, Karolinska Institutet, Stockholm, Sweden, ⁵Department of Haematology, Royal Bournemouth Hospital, Bournemouth, and ⁶WIMM, John Radcliffe Hospital, Oxford, UK.

E-mail: jacqueline.boultwood@ndcls.ox.ac.uk

Keywords: NPM1, myelodysplastic syndromes, del(5q).

First published online 15 April 2011
doi:10.1111/j.1365-2141.2011.08672.x

References


Gains of MYC locus and outcome in patients with diffuse large B-cell lymphoma treated with R-CHOP

The MYC oncogene is one of the major regulators of normal and neoplastic B-cells (Klapproth & Wirth, 2010). The translocation t(8;14)(q24;q32) deregulates the gene by juxtaposing it to the immunoglobulin heavy chain gene (IGH@) promoter and it is the most typical lesion of Burkitt lymphoma (Klapproth & Wirth, 2010; Lenz & Staudt, 2010). MYC can also be deregulated by chromosomal translocations in diffuse large B-cell lymphoma (DLBCL) (Boerma et al., 2009; Klapproth & Wirth, 2010; Lenz & Staudt, 2010; Auken et al., 2011). Two recent studies demonstrated that MYC translocations are significantly associated with worse survival in patients with DLBCL treated with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone) chemoimmunotherapy (Savage et al., 2009; Barrans et al., 2010). In a series of 245 DLBCL patients treated with the standard regimen R-CHOP, 35 cases (14%) presented a MYC rearrangement (Barrans et al., 2010). The overall survival (OS) at 2 years was 35% for patients with MYC rearrangement versus 61% for the remaining individuals (Barrans et al., 2010). Very similar data had been previously reported in a series of 135 DLBCL patients treated with R-CHOP (Savage et al., 2009): a 5-year OS of 33% for the 12 DLBCL cases (9%) bearing MYC rearrangement versus 72% for the remaining patients (Savage et al., 2009). Both papers suggest including the evaluation of MYC status in
the diagnostic panel for newly diagnosed DLBCL (Savage et al, 2009; Barrans et al, 2010). Whilst the clinical significance of the presence of MYC rearrangements appears well established, the clinical implications of extra copies of the gene are less clear. Stasik et al (2010) examined the significance of increased MYC gene copy number (CN) in a small series of 30 DLBCL cases, of which only 13 were treated with R-CHOP. No statistical differences in outcome were observed, but the eight cases with MYC gains had a 2-year OS of 43% compared to 73% in the 22 cases without increased MYC CN (Stasik et al, 2010). Previously, both the presence of MYC translocations and gains have been associated with a poorer outcome in a series of 156 patients with DLBCL treated with CHOP (Yoon et al, 2008).

With the aim of assessing the prognostic role of MYC gains in the R-CHOP era, we have investigated a series of 166 cases of DLBCL treated with R-CHOP and analysed by array-based comparative genomic hybridization (Scandurra et al, 2010). Gains affecting the MYC locus were detected in 17/166 (10%) patients, a percentage comparable to MYC translocations and to that previously reported (Yoon et al, 2008; Savage et al, 2009; Barrans et al, 2010). No patient had more than four copies of the gene. Cases with MYC locus gain expressed MYC mRNA at high levels, as evaluated by gene expression profiling with the Affymetrix U133 plus 2.0 arrays, although not significantly more than cases without MYC gain (Fig 1A). Gain at the MYC locus was never the only aberration and was statistically associated with the presence of other aberrations, such as gains at 13q31-3, 7p/7q, 1q, 9q, 2p16-p15, 12p/12q, 5p/5q and losses at 8p and 17p (Fig 1B, Table I). The pattern was

![Fig 1. Role of MYC locus gains in DLBCL: (A) levels of MYC expression in patients without and with MYC gain, as evaluated with Affymetrix U133 plus 2.0. RMA, Robust Multiarray Average; (B) Frequency plots of DNA gains (up) and losses (down) observed in 17 patients with a gain affecting the MYC locus and with a locus diploid status (X-axis, chromosome localization and physical mapping; Y-axis, percentage of cases bearing the aberration); (C) Kaplan–Meier estimates of overall survival (OS) according to the presence or absence of MYC gain with or without concomitant del(8p).](image)

<table>
<thead>
<tr>
<th>Associated region</th>
<th>MYC gain (8q24) (%)</th>
<th>No MYC gain (8q24) (%)</th>
<th>P</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 7p22.3–q36.3</td>
<td>59</td>
<td>20</td>
<td>0.001</td>
<td>0.023</td>
</tr>
<tr>
<td>+ 1q</td>
<td>41</td>
<td>10</td>
<td>0.0012</td>
<td>0.037</td>
</tr>
<tr>
<td>+ 9q</td>
<td>24</td>
<td>3</td>
<td>0.0051</td>
<td>0.041</td>
</tr>
<tr>
<td>+ 2p16.1–p15</td>
<td>47</td>
<td>17</td>
<td>0.0087</td>
<td>0.054</td>
</tr>
<tr>
<td>+ 5</td>
<td>24</td>
<td>3</td>
<td>0.0162</td>
<td>0.054</td>
</tr>
<tr>
<td>+ 12</td>
<td>29</td>
<td>7</td>
<td>0.0162</td>
<td>0.065</td>
</tr>
<tr>
<td>+ 11</td>
<td>24</td>
<td>4</td>
<td>0.0162</td>
<td>0.067</td>
</tr>
<tr>
<td>del(13q14.2–q21.31)</td>
<td>35</td>
<td>3</td>
<td>0.0162</td>
<td>0.010</td>
</tr>
<tr>
<td>del(8p)</td>
<td>29</td>
<td>4</td>
<td>0.0162</td>
<td>0.034</td>
</tr>
<tr>
<td>del(17p13.3–p11.2)</td>
<td>29</td>
<td>10</td>
<td>0.0162</td>
<td>0.122</td>
</tr>
</tbody>
</table>
similar to that reported for lesions concurrent with MYC translocations in Burkitt lymphoma and for lesions common in DLBCL of the germinatal centre B-cell type (Boerma et al., 2009; Lenz & Staudt, 2010). Three cases with concomitant MYC gain and translocation, as demonstrated by fluorescence in situ hybridization (FISH) using commercially available break-apart probes, were discarded from further analyses. After a median follow-up of 4.9 years (25th–75th percentiles ranging from 4 to 7 years), cases with MYC gain were associated with a poor OS ($P = 0.0012$) and progression-free survival ($P = 0.0053$) only in the presence of concomitant del(8p) (Fig 1C). Otherwise, no significant differences in outcome were observed (Fig 1C).

In conclusion, there are clear indications from the literature for the potential benefit of including the evaluation of MYC gene status by FISH in the diagnostic panel of newly diagnosed DLBCL patients. However, our data showed that the detection of extra copies of MYC did not determine a poor outcome in DLBCL patients treated with R-CHOP. Thus, proper attention should be paid by clinicians to the type of lesion reported, especially if patient-tailored therapeutic plans are to be considered. Prospective evaluation of MYC status in large series of cases will clarify the role of gains versus translocations in predicting a poor outcome in DLBCL patients.

Acknowledgements

MT interpreted data and co-drafted the manuscript. TCG, SMM, JV, WCC, AC, LB, AJMF, GG, collected and characterized samples and critically revised the manuscript. IK and MM analysed data and critically revised the manuscript. FB designed the research, analysed and interpreted data, co-drafted the manuscript. All Authors approved the manuscript.

We would like to thank our Colleagues: Giorgio Inghirami (Turin, Italy), Maurilio Ponzoni (Milan, Italy), Silvia Franceschetti (Novara, Italy), Miguel A. Piris (Madrid, Spain), Fabio Facchetti and Alessandra Tucci (Brescia, Italy), Silvia Uccella, Graziella Pinotti, Maria Grazia Tibiletti (Varese, Italy), Giovannii Martinelli and Giancarlo Pruner (Milan, Italy), Thierry Lazure and Olivier Lambotte (Paris, France), Josep Fr. Nomdedeu (Barcelona, Spain) for providing additional material and clinical information; Afua Adjeiwaa Mensah (Bellinzona, Switzerland) for manuscript editing. Work supported by: Oncosuisse grant OCS-1939-8-2006; Cantone Ticino (‘Computational life science/Ticino in rete’ program); Fondazione per la Ricerca e la Cura sui Linfomi (Lugano, Switzerland). M.M. was recipient of fellowship from Alto Adige Bolzano-AIL Onlus.

Monica Testoni1
Ivo Kwee1,2
Timothy C. Greiner3
Santiago Montes-Moreno4
Julie Vose5
Wing C. Chan3
Annalisa Chiappella5
Luca Baldini6
Andrés J.M. Ferreri7
Gianluca Gaidano8
Michael Mian1,9
Emanuele Zucca1
Francesco Bertoni1

1Laboratory of Experimental Oncology and Lymphoma Unit, Oncology Institute of Southern Switzerland (IOSI), Bellinzona, Switzerland, 2Dalle Molle Institute for Artificial Intelligence (IDSIA), Manno, Switzerland, 3Department of Pathology and Microbiology, University of Nebraska, Omaha, NE, USA, 4Molecular Pathology Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, 5Division of Haematology, San Giovanni Battista Hospital and University, Turin, Italy, 6Haematology/Bone Marrow Transplantation Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy, 7Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele H Scientific Institute, Milan, Italy, 8Division of Haematology, Department of Clinical and Experimental Medicine & BRMA, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy, and 9Division of Haematology, Azienda Ospedaliera S. Maurizio, Bolzano/Bozen, Italy

E-mail: frbertoni@mac.com

Keywords: malignant lymphomas, lymphomas, MYC, Array-CGH.

First published online 13 April 2011
doi:10.1111/j.1365-2141.2011.08675.x

References

Expression of PAX5 splice variants: a phenomenon of stress-induced, illegitimate splicing?

Extensive alternative splicing of PAX5 has been described in both malignant and normal B-cells and the existence of disease-specific isoforms has been suggested (Santoro et al., 2009). Disturbingly, the PAX5 isoform expression patterns observed were inconsistent between studies, even in normal B-cells. Thus, it has been questioned whether these variations result from an irreproducible pattern of PAX5 alternative transcripts or distinct expression patterns that are associated with specific B-cell malignancies (Borson et al., 2002; Robichaud et al., 2004; Sadakane et al., 2007; Sekine et al., 2007). While a recent study did not reveal any significant differences in the PAX5 transcript patterns between normal and cancerous B-lymphocytes derived from chronic lymphocytic leukaemia and lymphoma (Arseneau et al., 2009), others claim that either a higher number or even leukaemia-specific isoforms are expressed in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) (Santoro et al., 2009).

Our own observation – that paediatric BCP-ALL bone marrow (BM) samples which had been shipped overnight expressed higher numbers of isoforms – and previous studies suggesting that ‘ageing’ of blood or cold shock conditions may induce illegitimate splicing (Gayther et al., 1997; Ars et al., 2000; Wimmer et al., 2000; Liu et al., 2010) prompted us to investigate the expression of PAX5 isoforms under such stress conditions.

The analysis of PAX5 isoforms in cells isolated from freshly drawn blood from healthy donors as compared to those of ‘aged’ lymphocytes isolated from blood which was left at room temperature (RT) for 1–4 d (for details see Appendix S1), irrespective of the anticoagulant used, reproducibly revealed a progressive increase of alternative splice variants at the expense of full-length (FL) PAX5 transcripts (Fig 1A, B). Both alternative FL-PAX5 transcripts comprising exons 1A-10 or exons 1B-10 (Fig 1A, B) were affected in the same way, and separate amplification of either the 5’ region (exons 1A-6) or the 3’ region (exons 5–10) of PAX5 (Fig 1C, D) verified this observation.

Although in fresh samples inter-individual differences in the PAX5 expression patterns were found, FL-PAX5 was always the predominant isoform. In our hands also the cell lines NALM6 and REH expressed mainly FL-PAX5, no matter which culture conditions, including starvation and suboptimal cell densities (for details see Appendix S1), were used (data not shown). However, incubation of the B-cell line RAJI at RT for 24 or 48 h induced the expression of PAX5 isoforms and subsequent re-cultivation at 37°C reversed this process (Fig 1E), supporting the hypothesis of cold shock-stimulated illegitimate splicing. These data corroborate the recent finding that FL-PAX5 is always the predominant isoform (Arseneau et al., 2009), but contradict earlier studies in which PAX5 variants, rather than FL-PAX5, were expressed in some B-cell lines (Robichaud et al., 2004; Arseneau et al., 2009).

Cloning and sequencing of a number of splice variants amplified from ‘aged’ normal lymphocytes revealed that all isoforms identified corresponded to those previously described (Borson et al., 2002; Robichaud et al., 2004; Arseneau et al., 2009), including transcripts with various deletions of exons such as Δ2, Δ7/8, Δ2/7/8, Δ2/7/8/9, Δ2/4/8, and Δ2/3/4/8. Importantly, also the PAX5.12 isoform, which has been recently suggested to play a role in the development of BCP-ALL (Santoro et al., 2009), was readily expressed in ‘aged’ normal lymphocytes.

The basis for age- and temperature-induced missplicing has not been studied in detail yet. Nevertheless, it has been proposed that the fidelity of splicing – at least for some genes – is affected in ‘aged’ lymphocytes (Wimmer et al., 2000; Liu et al., 2010), rather than caused by a general RNA degradation. Accordingly, the transcript patterns of HPRT1, IKZF1, and RUNX1 were not affected by ‘ageing’ of the samples (Fig 1F–H). Together, these data strongly suggest that in ‘aged’ blood, PAX5 is prone to abnormal isoform expression.

To further substantiate that PAX5 isoform expression patterns in BCP-ALL may be altered dependent on the elapsed...
Bibliography
Bibliography


Bibliography


Bibliography


Bibliography


353. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP.
List of publications

A) ORIGINAL PAPERS:


B) ABSTRACTS:


**Curriculum Vitae**

**First name**  Monica  
**Surname**  Testoni  
**Date of birth**  November 19th, 1984  
**Place of birth**  Como, Italy  
**Citizenship**  Italian  
**Marital status**  unmarried  
**Home address**  Via Prestino 40, 22100 Como, Italy  
**Work address**  Lymphoma & Genomics Research Program, IOR - Institute of Oncology Research  
via Vincenzo Vela 6, 6500 Bellinzona, Switzerland  
**Work phone**  +41 91 820 0363  
**fax**  +41 91 820 03 05  
**Mobile phone**  +39 333 8651970  
**E-mail**  monica.testoni@gmail.com; monica.testoni@ior.iosi.ch  

**EDUCATIONAL BACKGROUND:**

- **April 22nd, 2010**  Master’s degree in Biology, specialized in functional and molecular biology, State University of Milan-Bicocca, Milan, Italy  
- **November 14th, 2007**  Bachelor’s degree in Biotechnology, specialized in molecular biotechnology, State University of Milan-Bicocca, Milan, Italy  

**PRACTICAL SKILLS:**

**MOLECULAR BIOLOGY:**  Experienced with many molecular biology techniques on DNA, RNA and protein such as nucleic acid and protein extractions, PCR, RT-PCR; cloning and sequencing; Western Blot, Chromatin Immunoprecipitation (ChIP); basic data mining of Affymetrix and Illumina DNA and RNA arrays (Partek, IGV, GSEA and MetaCore).  

**CELLULAR BIOLOGY:**  Experience with many lymphoma cell lines; stable and transient transfection techniques; lentivirus production and infection, FACS analysis [cell cycle, apoptosis, proliferation assay, detection of surface markers]; cellular toxicity assay (MTT assay); limited in vivo experience;
WORKING EXPERIENCE:

Since May 2010  PhD student, registered at the University of Geneva (Switzerland), at IOR - Institute of Oncology Research (former “Laboratory of Experimental Oncology, IOSI - Oncology Institute of Southern Switzerland”, up to 9-2011), Bellinzona, Switzerland.

March 2009 – April 2010  Internship for Master’s degree at the Laboratory of Experimental Oncology, IOSI - Oncology Institute of Southern Switzerland, Bellinzona, Switzerland

September - December 2006  Internship at Medical Oncology Division, San Paolo Hospital, Milan

HONORS AND FELLOWSHIPS:

2010  Master’s Degree in Biology, Italian grade 108/110, “Array-CGH in DLBCL identifies new genes involved in lymphomagenesis” (State University of Milano-Bicocca, April 2010) (written in English).

Internal supervisor: Prof. Antonella Ronchi
External supervisor: Dr. Francesco Bertoni

2007  Bachelor’s degree in Biotechnology, Italian grade 97/110, “Regulative answers to iron homeostasis in mammal cells” (State University of Milano-Bicocca, November, 2007)

Supervisor: Prof. Giovanna Lucchini