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The role of EGR-1 and calcium influx in the antitumor activity of anti-CD20 monoclonal antibodies

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LE ROLE D'EGR-1 ET DU FLUX CALCIQUE DANS L'ACTIVITE ANTITUMORALE DES ANTICORPS MONOCLONAUX ANTI-CD20

Les anticorps monoclonaux (AcM) anti-CD20 sont essentiels pour le traitement du lymphome non hodgkinien et de la leucémie lymphoïde chronique (LLC). Les AcM agissent soit en activant directement la signalisation apoptotique dans les cellules cibles, soit via le système immunitaire. Dans une étude préclinique, nous avons montré que le traitement avec AcM anti-CD20, rituximab et GA101, induit l'expression de la protéine early growth response 1 (EGR-1) (*Dalle et al., 2011*). EGR-1 est un facteur de transcription régulé par le calcium (Ca^{2+}) et CD20 est impliqué dans la régulation du flux calcique transmembranaire. Nous avons donc étudié le rôle d'EGR-1 et du flux Ca^{2+} dans l'activité cytotoxique des AcM anti-CD20. Nous avons montré qu'EGR-1 est rapidement induit suite à l'exposition au rituximab et à GA101. La baisse de l'expression d'EGR-1 par shRNA a supprimé l'effet cytotoxique du GA101 à la fois *in vitro* et *in vivo*, indiquant qu'EGR-1 est requis pour la mort cellulaire médiée par CD20. De plus, la surexpression d'EGR-1 augmente la sensibilité au GA101 *in vitro* et *in vivo*. En outre, nos résultats indiquent que les AcM anti-CD20 induisent un flux Ca^{2+}. Le blocage du flux Ca^{2+} par inhibiteurs de canaux calciques (ICC) a aboli l'induction d'EGR-1 ainsi que l'efficacité du GA101 *in vivo* et *ex vivo* dans des échantillons de LLC. Plus important, nos données indiquent que les patients recevant des ICC ont une moins bonne réponse au traitement par les AcM anti-CD20. En conclusion, nous avons identifié EGR-1 comme potentiel biomarqueur pour prédire la réponse à la thérapie anti-CD20 et démontré que les ICC ont un impact négatif sur l'efficacité des AcM anti-CD20 chez les patients.

Mots clés : Anticorps monoclonaux anti-CD20 ; rituximab ; GA101 ; EGR-1 ; flux calcique ; Inhibiteurs des canaux calciques ;

THE ROLE OF EGR-1 AND CALCIUM INFLUX IN THE ANTITUMOR ACTIVITY OF ANTI-CD20 MONOCLONAL ANTIBODIES

Anti-CD20 monoclonal antibodies (mAbs) are an essential component of the treatment of patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL). They mediate their antitumor effects by activating the immune system or by direct apoptotic signaling in target cells. In a previous preclinical study, we showed that treatment with anti-CD20 mAbs, rituximab and GA101, resulted in upregulated expression of early growth factor 1 (EGR-1) (Dalle et al. 2011). EGR-1 is a calcium (Ca2+) regulated transcription factor and CD20 is hypothesized to regulate transmembrane Ca2+ flux. Therefore, we aimed to assess the role of EGR-1 and Ca2+ flux in the cytotoxic activity of anti-CD20 mAbs. We have shown that EGR-1 expression is rapidly upregulated in CD20+ cells following rituximab and GA101 exposure. Decreasing EGR-1 expression by shRNA abolishes the direct cytotoxic effect of GA101 both in vitro and in vivo, indicating that EGR-1 is required for CD20-mediated cell death. Additionally, the overexpression of EGR-1 enhances the cytotoxic activity of GA101 both in vitro and in vivo. Furthermore, our results indicate that anti-CD20 mAbs induce calcium influx. Blocking the Ca2+ flux with calcium channel blockers (CCB) abolishes EGR-1 induction and impaires the GA101 efficacy in vivo and ex vivo in CLL blood samples. More importantly, our data indicate that patients receiving CCBs and anti-CD20 therapy have worst progression free survival and overall survival. In conclusion we have identified EGR-1 as a potential biomarker to predict response to anti-CD20 therapy. We demonstrated that co-treatement with CCBs negatively impacts the outcome of patients receiving anti-CD20 mAbs.

Key words: Anti-CD20 monoclonal antibodies; rituximab; GA101; EGR-1; Calcium influx; Calcium channel blockers;

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FRENCH SUMMARY

Les hémopathies malignes B regroupent une grande diversité de pathologies lymphoprolifératives provenant de lymphocytes B à différents stades du développement. Ils incluent la plupart des lymphomes non-hodgkiniens (LNH), certaines leucémies B et myélomes. Les lymphomes non hodgkiniens sont des tumeurs solides du système immunitaire, représentant environ 90% de tous les lymphomes. Les LNH englobent un groupe hétérogène de cancers, dont la majorité provient de cellules B (80-85%) et le reste provient de cellules T ou NK. La leucémie lymphoïde chronique (LLC) fait partie des tumeurs à cellules lymphoïdes B matures. La LLC se caractérise la multiplication et l'accumulation de cellules lymphoïdes anormales dans le sang, les ganglions, la rate et la moelle osseuse. Le traitement du NHL et LLC est dans la plupart des cas une combinaison de plusieurs approches, y compris la chimiothérapie cytotoxique conventionnelle, les thérapies ciblées ou l'immunothérapie. L'immunothérapie sous la forme d'anticorps monoclonaux dirigée contre le cluster de différenciation présent à la surface des lymphocytes B - CD20, a considérablement fait progresser le traitement des patients atteints d'hémopathies malignes. Le rituximab, anticorps monoclonal dirigé contre le CD20 a constitué le premier anticorps commercialisé dans une indication oncologique. Il existe actuellement d'autres anticorps dirigés contre cette même cible, dont le GA101 (ou obinutuzumab) qui a récemment été approuvé pour le traitement de la LLC. Les mécanismes d'action des anticorps qui agissant désormais soit directement sur la cellule tumorale, soit sur le microenvironnement et le système immunitaire, sont très complexes. A ce jour les mécanismes d'actions du rituximab et du GA101 et les voies de signalisation mises en jeu après liaison avec le CD20 restent peu explorés.

Notre équipe a eu l'opportunité d'analyser l'effet de ces deux anticorps, rituximab et GA101, dans des modèles précliniques et de démontrer leur implication dans la modulation de l'expression de certains gènes, suggérant leur implication dans les mécanismes de cytotoxicité de ces deux molécules. Parmi ces gènes induits par les anticorps anti-CD20, *early growth response -1*

(*EGR-1*) voit son expression fortement induite. EGR-1 code pour un facteur de transcription a doigts de zinc, dont l'expression est fortement régulée par diffèrent stimuli extracellulaire, y compris l'influx calcique. Par ailleurs, de nombreuses études indiquent l'implication du récepteur CD20 dans la régulation du flux calcique au niveau des lymphocytes B. Nous avons donc investigué le rôle d'EGR-1 et du flux calcique dans l'activité antitumorale des anticorps monoclonaux anti-CD20.

Nos résultats indiquent qu'EGR-1 est une protéine clé dans la modulation de l'apoptose par la signalisation CD20. Suite à l'exposition aux anticorps anti-CD20, nous observons une forte surexpression d'EGR-1 au niveau transcriptionnel et protéique. En parallèle, l'exposition aux anticorps anti-CD20 se traduit par une forte induction de la mort cellulaire, ce qui suggère une corrélation entre l'induction d'EGR-1 et l'effet cytotoxique des anticorps. En effet l'atténuation de l'expression d'EGR-1 par des shRNA se traduit par une suppression de l'effet cytotoxique de GA101, à la fois *in vitro* et *in vivo*, indiquant qu'EGR-1 est nécessaire pour la mort cellulaire médiée par CD20. De plus, cette observation est confortée par des résultats de surexpression d'EGR-1 par le biais d'un plasmide, qui se traduit par une augmentation la sensibilité au GA101 *in vitro* et *in vivo*. Aussi, nos résultats démontrent que l'exposition aux anticorps monoclonaux anti-CD20 se traduit par une augmentation du flux Ca2+ entrainant l'augmentation de l'expression d'EGR1. Par ailleurs, nous avons observé qu'en présence de nifedipine, un inhibiteur des canaux calciques, l'expression d'EGR-1 n'est plus induite par les anticorps anti-CD20. En effet, le blocage du flux calcique par des inhibiteurs de canaux calciques a aboli l'induction d'EGR-1 ainsi que l'efficacité du GA101 aussi bien *in vivo* qu'*ex vivo*, dans des échantillons de sang des patients atteints de LLC. Comme les inhibiteurs des canaux calciques sont souvent utilisés en clinique dans le traitement des maladies cardiovasculaires, nous avons investigué leur influence sur l'efficacité du traitement pas des anticorps anti-CD20. Les données obtenues de l'étude clinique GOYA, indiquent que les patients recevant des inhibiteurs des canaux calciques ont significativement une moins bonne réponse (survie sans progrès et survie globale) au traitement par les anticorps monoclonaux anti-CD20.

En conclusion, nos résultats ont permis d'identifier la protéine EGR-1 comme un biomarquer potentiel de la réponse à la thérapie utilisant des anticorps monoclonaux anti-CD20. Plus important, nous avons démontré que les inhibiteurs des canaux calciques ont un impact négatif sur l'efficacité des anticorps monoclonaux anti-CD20 chez les patients atteints d'hémopathies malignes B.

Les résultats obtenus permettent une meilleure connaissance des mécanismes impliqués dans l'action des anticorps monoclonaux et pourraient avoir de réelles conséquences en clinique dans la recherche de nouvelles approches thérapeutiques permettant la potentialisation des effets des anticorps ant-CD20.

TABLE OF CONTENTS

LIST OF FIGURES 11

LIST OF TABLES 12

LIST OF ABBREVIATIONS 13

PREFACE 16

INTRODUCTION 18

Chapter I: B-cell malignancies 19

1. Non-Hodgkin's lymphoma 19

1.1. Definition 19

1.2. Incidence and mortality 19

1.3. Risk factors 20

1.4. Pathophysiology 20

1.5. NHLs Classification 21

1.6. Clinical presentation and staging 22

1.7. Prognostic factors 22

2. Chronic Lymphocytic Leukemia 23

2.1. Definition 23

2.2. Incidence and mortality 23

2.3. Risk factors 24

2.4. Pathophysiology 24

2.5. Clinical presentation and staging 25

2.6. Prognostic factors 26

Chapter II: Therapeutic strategies for B-cell malignancies 27

1. Chemotherapy 27

2. Radiation therapy 28

3. Targeted small molecule inhibitors 29

3.1. Inhibitors of BCR signaling 29
3.1.1. Bruton tyrosine kinase inhibitors 29
3.1.2. Phosphatidylinositol 3-kinase inhibitors 30
3.1.3. Spleen tyrosine kinase 30
3.2. Bcl-2 family inhibitors 31
4. Immunotherapy 31
4.1. Monoclonal antibody therapy 31
4.2. Adoptive T cell therapies 32
4.3. Cytokines 33
Chapter III: Anti-CD20 monoclonal antibodies 34
1. Structure and development of monoclonal antibodies 34
2. Mechanisms of action 36
2.1. Direct cell death induction 37
2.2. Complement dependent cytotoxicity 37
2.3. Antibody-dependent cellular cytotoxicity 38
2.4. Antibody-dependent cellular phagocytosis 38
3. CD20, a target for mAbs 39
4. Rituximab 40
5. Obinutuzumab (GA101) 41
6. Ofatumumab 43
7. Ibritumomab tiuxetan 43
8. Tositumomab / I^{131} Tositumomab 43
9. Other anti-CD mAbs in clinical trials 44
9.1. Ocrelizumab 44
9.2. Veltuzumab 44
9.3. Ocaratuzumab 44
9.4. RhuMAb 45
9.5. Ublituximab 45

10. Resistance 45
Chapter IV Early Growth Response -1 47
1. Immediate early genes 47
2. EGR family of transcription factors 48
3. EGR-1 - a transcription factor in the crossfire of signal transduction cascades 49
3.1. Post-translational modification of EGR-1 51
3.2. EGR-1 and cancer 51
3.2.1. EGR-1 as a tumor suppressor gene 52
3.2.2. EGR-1 as an oncogene 53
3.3. EGR-1 in B cells 54
4. Early growth response -2 54
5. Early growth response – 3 55
6. Early growth response - 4 55
Chapter V Calcium signaling 57
1. Calcium channels 57
1.1. Voltage-gated Ca^{2+} channels 57
1.2. Non-voltage-gated Ca^{2+} channels 58
1.2.1. Ca^{2+} Release Activated Ca^{2+} channel 58
1.2.2. Store Independent Ca^{2+} Channels 60
2. Calcium entry 60
2.1. Capacitive Ca^{2+} entry 60
2.2. Constitutive Ca^{2+} entry 61
3. Role of calcium signaling 62
3.1. Cell proliferation 63
3.2. Transcription factor activation 63
3.3. Calcium and apoptosis 63
3.4. Ca^{2+} dysregulation and cancer 64

4. Calcium channel blockers 65
AIMS OF THE STUDY 67
RESULTS 68
DISCUSSION 113
CONCLUSION AND PERSPECTIVES 120
BIBLIOGRAPHY 122

LIST OF FIGURES

Figure 1. B-cell antigen receptor signaling in CLL..... 29
Figure 2. Structure of an antibody..... 35
Figure 3. Different mechanisms of action of type I and II anti-CD20 mAbs..... 36
Figure 4. Glycoengineered structure of GA101 42
Figure 5. Structure of the EGR transcription-factor family 48
Figure 6. Signaling pathways inducing Egr-1 expression. 50
Figure 7. Structure and post-translational modification of EGR-1..... 51
Figure 8. Molecular organization of voltage-gated Ca^{2+} channel subunits in the plasma membrane..... 58
Figure 9. The molecular choreography of CRAC channel activation 59
Figure 10. EGR-1 in human follicular lymphoma 112

LIST OF TABLES

Table 1. Non-Hodgkin's lymphoma classification..... 21
Table 2. Ann Arbor staging system of NHL. 22
Table 3. Rai staging system of CLL. 25
Table 4. Binet staging system of CLL. 25
Table 5. Chemotherapeutic agents used for the treatment of B-cell malignancies 28

LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cellular Phagocytosis
ARC	Arachidonate-Regulated Ca^{2+} channel
BCR	B-cell Receptor
BL	Burkitt's Lymphoma
BTK	Bruton Tyrosine Kinase
CAR	Chimeric Antigen Receptor
CCB	Calcium Channel Blocker
CDC	Complement Dependent Cytotoxicity
CLL	Chronic Lymphocytic Leukemia
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)
CR	Complete Response
CRAC	Ca^{2+} Release Activated Ca^{2+} channel
DLBCL	Diffuse Large B-cell Lymphoma
EBV	Epstein-Barr Virus
EGR	Early Growth Response
ER	Endoplasmic Reticulum
FDA	US Food and Drug Administration
Fab	Fragment antibody binding
Fc	Fragment Crystallizable
FL	Follicular Lymphoma

HCV	Hepatitis C Virus
HL	Hodgkin's Lymphoma
HIV	Human Immunodeficiency Virus
IEG	Immediate Early Gene
IFN	Interferon
IGHV	Immunoglobulin Heavy-Chain Variable region
IPI	International Prognostic Index
KO	Knock-out
mAb	Monoclonal Antibody
MAC	Membrane Attach Complex
MCL	Mantle Cell Lymphoma
MZL	Marginal Zone Lymphoma
NHL	Non-Hodgkin's Lymphoma
NK	Natural Killer
ORR	Overall Response Rate
PD1	Programmed Death-1
PFS	Progression-free Survival
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and Tensin homolog
ROC	Receptor Operated Channels
SIC	Store Independent Ca^{2+} Channels
SICE	Store-Independent Ca^{2+} Entry
SMOC	Second Messenger Operated Channels

SOCE	Store-Operated Ca^{2+} Entry
SOCS	Store Operated Channels
STIM1	Stromal Interaction Molecule 1
SYK	Spleen Tyrosine Kinase
TGFβ1	Transforming Growth Factor Beta 1
TRP	Transient Receptor Potential
WHO	World Health Organization

PREFACE

B-cell malignancy is a collective term for a heterogeneous group of lymphoproliferative malignancies, originating from B cells at different stages of B-cell development. They represent a diverse collection of diseases, including non-Hodgkin's lymphomas (NHL) and chronic lymphocytic leukemia (CLL). The treatment and management of B-cell malignancies has been revolutionized by the addition monoclonal antibodies (mAbs) to standard therapy regimens. Rituximab, the first mAb used in clinic, directed against the B cell antigen CD20, has significantly improved the treatment outcome of patients with B-cell malignancies. Following its success, new generation of anti-CD20 antibodies have been developed, among which the first glycoengineered antibody GA101 (obinutuzumab) with superior antitumor activity. Anti-CD20 mAbs achieve their therapeutic potential through several different mechanisms of action, inducing the antibody-dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and direct cell death induction. While ADCC and CDC have been widely studied and are likely to be clinically relevant, the direct cell death induction and the signaling pathways involved have not been fully elucidated.

In the Introduction of this thesis, I will present an overview on B cell malignancies and the different therapeutic modalities. The following part focuses on monoclonal antibodies, in particular anti-CD20 monoclonal antibodies. Next, I will describe the early growth response family of transcription factors, emphasizing early growth response-1. Finally, in the last chapter, the calcium flux and calcium channel blockers will be evoked in relationship to cancer.

In the Results section, I will present the work performed during my PhD study, which shows the importance of EGR-1 and calcium influx in the direct cell death induction by rituximab and GA101.

In the final sections, Discussion, Conclusion and Perspectives, I will summarize our work and discuss the objectives for future studies. Overall, our

results show that EGR-1 and calcium influx are required for the induction of direct cell death by anti-CD20 mAbs. Additionally, our results indicate that calcium channel blockers impair the efficacy of anti-CD20 mAbs.

INTRODUCTION

Chapter I:
B-cell malignancies

B-cell malignancy is a collective term for a heterogeneous group of lymphoproliferative malignancies, originating from B cells at different stages of B-cell development. [1] They represent a diverse collection of diseases, including non-Hodgkin's lymphomas (NHL), B-cell leukemias and myeloma.

1. Non-Hodgkin's lymphoma

1.1. Definition

Non-Hodgkin's lymphomas are solid tumors of the immune system, accounting for about 90% of all lymphomas, and the remaining 10% are referred to as Hodgkin's lymphomas (HL). NHLs encompass a heterogeneous group of cancers, the majority of which arise from B cells (80-85%) and the remainder derive from T or NK cells. [2]

1.2. Incidence and mortality

NHL is the seventh most frequently diagnosed form of cancer in the United States, accounting for approximately 4,3 % of all cancers, with an estimated 72,000 new cases (19,5 per 100,000 men and women) per year. NHL is also the eighth leading cause of cancer death in the United States, with approximately 5,9 per 100,000 men and per year. [3] Approximately 2.1 percent of men and women will be diagnosed with NHL at some point during their lifetime. More than two-thirds of patients diagnosed with NHL are 60 years and older.

For the past 20 years, the incidence of non-Hodgkin's lymphoma has been rising steadily across all age groups and in both sexes, by 3-5% per year. [4] The reason for this increase is unclear, although the increasing age of the

population causes an additional increase in the total number of patients with NHL. [4,5]

1.3. Risk factors

The most clearly defined risk factor for the development of non-Hodgkin's lymphoma is immuno-suppression. Patients with human immunodeficiency virus (HIV) infection have an increased risk of developing non-Hodgkin's lymphoma. The risk is increased in post-organ-transplant immune deficiencies, as well as in patients with inherited immunodeficiency syndromes. [6] Infection also plays a part in the development of some lymphomas, either by inhibition of immune function or by induction of chronic inflammatory response. Epstein-Barr virus (EBV), has recognized associations with the development of Burkitt's lymphomas (BL). [7] Other microorganisms have been shown to be associated with the development of specific lymphomas such as hepatitis C virus (HCV) with splenic marginal-zone lymphoma, Borrelia burgdorferi with cutaneous mucosa-associated lymphoid tissue lymphoma, and Helicobacter pylori infection with gastric mucosa-associated lymphoid tissue lymphoma. [8,9]

1.4. Pathophysiology

NHLs represent a heterogeneous group of malignancies that arise from B-cells at various stages of differentiation. Similar to other types of cancer, NHLs develop from the multistep accumulation of genetic aberrations that induce a selective growth advantage of the malignant clone. Recurrent translocations, which occur during different steps of B-cell differentiation, are often an initial step in the malignant transformation. These translocations lead to deregulated expression of oncogenes that often control cell proliferation, survival, and differentiation. [10,11] Interestingly, these translocations alone are often insufficient for lymphoma development. Accordingly, secondary genetic alterations are required for the full malignant transformation.

1.5. NHLs Classification

NHL is a diverse group of malignancies which usually develops in the lymph nodes, but can occur in any tissue. Several different classification systems have been proposed that have grouped these malignancies according to their histological characteristics. The most recent system is the fifth edition of the World Health Organisation (WHO) classification of tumors of haemopoietic and lymphoid tissues, published in 2016 (table 1). [12] More than 70 specific NHL subtypes have been identified by the WHO. NHL subtypes are categorized by the characteristics of the lymphoma cells, including their morphology, the presence of proteins on the surface of the cells and their genetic features. [12,13]

NHL subtype	Epidemiology	Genetic abnormalities
Follicular lymphoma	20-30 % of adult NHL	Bcl-2
Marginal zone lymphoma	9% of adult NHL	Myd88
Mantle cell lymphoma	5-10 % of adult NHL	Bcl-1, cyclin D1
Diffuse large B-cell lymphoma	30-40 % of adult NHL	Bcl-2 (15-30%), Bcl-6 (20-40%), c-myc (5-15%)
Burkitt lymphoma	1 % of adult NHL	c-myc, Bcl-6,
T-cell lymphoma	5-10 % of adult NHL	Tet2, Dnmt3A, Idh2, RhoA, CD28

Table 1. Non-Hodgkin's lymphoma classification

The NHL subtypes are also characterized according to how the disease progresses:

- Aggressive lymphomas account for about 60 percent of all NHL cases. Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive NHL subtype.

- Indolent lymphomas evolve more slowly with fewer signs and symptoms when first diagnosed. Slow-growing or indolent subtypes represent about 40 percent of all NHL cases. Follicular lymphoma (FL) is the most common subtype of indolent NHL.

1.6. Clinical presentation and staging

Clinical presentation is dependent on the site of involvement, natural history of the lymphoma subtype, and presence or absence of B symptoms (weight loss >10%, night sweats, body temperature >38˚C). Based on these criteria, NHL is staged using the Ann Arbor classification system according to the principal stages as described in Table 2.

Principal stages	
I	Involvement of one lymph node or one extranodal organ or site (IE)
II	Involvement of two or more lymph node regions on the same side of the diaphragm, or localised involvement of an extranodal site or organ (IIE) and one or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph node regions on both sides of the diaphragm, which might be accompanied by localised involvement of an extranodal organ or site (IIIE) or spleen (IIIS) or both (IIISE); the spleen is regarded as nodal
IV	Diffuse or disseminated involvement of one or more distant extranodal organs with or without associated lymph node involvement

Table 2. Ann Arbor staging system of NHL.
Adapted from Shankland et al., 2012

1.7. Prognostic factors

The International Prognostic Index (IPI) is the most widely used prognostic model for patients with NHL. [14,15] It takes into account age (≤60 years vs.>60 years), serum lactate dehydrogenase concentration (normal vs abnormal), Ann Arbor stage (I/II vs. III/IV), Eastern Cooperative Oncology Group performance status (<2 vs. ≥2) and number of extranodal sites involved

(≤one vs.>one). This score has been used to define four risk groups: low risk (zero to one clinical feature), low-intermediate risk (two features), high-intermediate risk (three features), and high risk (four to five features). When applied to a large cohort of patients, these risk groups had 5-yearsurvival rates of 73%, 51%, 43%, and 26%, respectively.

Age is a particularly important prognostic marker, and has been repeatedly associated with poorer outcomes. However, elderly patients who are able to receive full-dose chemotherapy have survival rates similar to their younger counterparts.

2. Chronic Lymphocytic Leukemia

2.1. Definition

Chronic lymphocytic leukemia (CLL) is a B cell malignancy that is characterized by the accumulation of small, mature-appearing neoplastic lymphocytes in the blood, bone marrow and peripheral lymphoid organs. [16]

2.2. Incidence and mortality

CLL is the most common adult leukemia in western countries, whereas it is relatively rare in Asia. In the United States, CLL accounts for approximately 1.2 % of all cancers, and with an estimated 20,110 new cases (4.3 per 100,000 men and women) per year. Death rates, 1.3 per 100,000 men and women per year, are higher among older adults, or those 75 and older. [17] The risk of developing CLL increases with age; the median age at diagnosis ranges from 70 to 72 years.

2.3. Risk factors

Inherited susceptibility to chronic lymphocytic leukemia (CLL) has been recognized for decades. Approximately 12% of patients with CLL report a family history of a lymphoproliferative disorder, and 6–9% have a relative who also has CLL.[18] Asians have significantly lower rates of CLL than Caucasian–Americans or Europeans and, importantly, those low rates are maintained even among Asian people who immigrate to Western counties, suggesting that genetic rather than environmental factors are critical in these differences. [19] Nevertheless, the US Department of Veterans Affairs has accepted that exposure to Agent Orange is a risk factor for CLL [20], and several lines of evidence suggest that exposure to insecticides might be a risk factor for CLL.[21] By contrast, no evidence suggests that dietary or lifestyle factors increase the risk of CLL.

2.4. Pathophysiology

Several large genetic studies have revealed numerous genetic alterations in CLL, including chromosomal alterations, mutations, alterations in the expression of miRNAs and epigenetic modifications. Approximately 80% of patients with CLL carry a chromosomal alteration: either a deletion in one of their chromosome 13q (55%), 11q (18%), 17p (7%), 6q (7%) or have a trisomy 12 (16%). [22] Additionally, the presence of recurrent somatic mutations have been consistently observed in genes that have a role in DNA damage (for example, TP53 and ATM), mRNA processing, chromatin modification, WNT signaling, Notch signaling and inflammatory pathways (MYD88). [16] As such, mutations in these genes can lead to a range of cellular consequences, such as aberrant DNA repair and B cell receptor (BCR) signaling. A functional BCR is required for the survival of mature B cells and is maintained in most mature B cell malignancies, including CLL. Additionally, interactions between CLL cells and their microenvironment, including interactions with other cell types, such as T cells, nurse-like cells and stromal cells, can induce B cell proliferation and contribute to disease.

2.5. Clinical presentation and staging

Two clinical staging systems are widely used to divide patients with CLL into three broad prognostic groups. The Rai staging system (Table 3) is more commonly used in the United States, whereas the Binet classification (Table 4) is more commonly used in Europe. The staging systems each recognize the importance of bone marrow function and define late-stage, or high-risk, disease by the presence of pronounced anemia or thrombocytopenia.

Risk group	Clinical features
Low risk (Rai stage 0/I)	Lymphocytosis without cytopenia, lymphadenopathy or splenomegaly
Intermediate risk (Rai stage II)	Lymphocytosis, lymphadenopathy and/or splenomegaly, but without cytopenia
High risk (Rai stage III/IV)	Lymphocytosis and cytopenia (a haemoglobin level of ≤11 g per dl and/or a platelet count of ≤ 100,000 cells per µl)

Table 3. Rai staging system of CLL.
Adapted from Kipps et al., 2017

Risk group	Clinical features
Low risk (Binet stage A)	Less than three palpably enlarged sites‡ without cytopenia
Intermediate risk (Binet stage B)	Three or more palpably enlarged sites‡ without cytopenia
High risk (Binet stage C)	Cytopenia (a haemoglobin level of ≤10 g per dl and/or a platelet count of ≤100,000 cells per µl)

Table 4. Binet staging system of CLL.
Adapted from Kipps et al., 2017

2.6. Prognostic factors

The clinical course of newly diagnosed CLL is extremely variable. Some patients remain free of symptoms and are fully active for decades, whereas others rapidly become symptomatic or develop high-risk disease, which requires treatment soon after diagnosis.

While an increasing number of elderly patients are being diagnosed with early stage disease, the therapeutic options of younger patients with CLL has evolved. In selected patient, an adapted prognostic index, the CLL-IPI combines genetic, biochemical, and clinical parameters including: TP53 status, IGHV mutational status, serum β2-microglobulin concentration (≤3·5 mg/L vs >3·5 mg/L), clinical stage and age (≤65 years vs >65 years). [23] Based on these prognostic factors, four risk groups have been identified with significantly different overall survival at 5 years: low (93.2%), intermediate (79.3%), high (63.3%), and very high risk (23.3%).

Chapter II:
Therapeutic strategies for B-cell malignancies

The treatment of patients with B-cell malignancies can include chemotherapy, a combination of chemotherapy and immunotherapy, or small molecules that target the signaling pathways that promote the growth and/or survival of malignant cells. Radiotherapy is used in a limited number of situations.

1. Chemotherapy

Cytotoxic chemotherapy is a systemic treatment that uses drugs to stop the growth of cancer cells, leading to cell death. Chemotherapy is administrated repeatedly (cycles) and may be given by mouth, injection, or infusion. Currently, it is the first-line treatment for B-cell malignancies, and it is administered in combination to maximize cell killing while minimizing overlapping toxicity. The chemotherapy regimen used depends on the stage and type of NHL. The most common chemotherapy combination for the initial treatment of aggressive NHL is called CHOP and contains four drugs: cyclophosphamide, hydroxyl-daunomycin (doxorubicin), Oncovin® (vincristine), and prednisone. There are other common combinations of chemotherapy regimens as well, including: bendamustine, fludarabine, cyclophosphamide, etoposide, platinum compounds. Examples of chemotherapeutic molecules that are commonly used for NHL treatment are listed in table 5 according to their mechanisms of action.

Mechanism of action		Example
Modification of DNA structure	Formation of covalent bonds with DNA	Alkylating agents: cyclophosphamide ifosfamide bendamustine
	DNA break	bleomycin
	Intercalating agents	Anthracyclins: doxorubicin, daunorubicin
Antimetabolites	Inhibition of nucleic acid synthesis	fludarabine, cladribine, gemcitabine cytarabine, gemcitabine, methotrexate
Mitotic poisons	Destruction of mitotic spindles	Vinca alkaloids: vincristine, vinblastine, vinorelbine

Table 5. Chemotherapeutic agents used for the treatment of B-cell malignancies

2. Radiation therapy

Radiation therapy is the use of high-energy x-rays, electrons, or protons to destroy cancer cells. A radiation therapy regimen usually consists of a specific number of treatments given over a set period of time. It is usually given after or in addition to chemotherapy, depending on the NHL subtype. It is most often given to patients who have lymphoma that is localized, meaning it involves only 1 or 2 adjacent areas, or who have a lymph node that is particularly large. It may also be given for the treatment of pain or in very low doses (just 2 treatments) to patients with advanced disease who have localized symptoms that can be relieved using radiation therapy. However, the role of

radiation therapy continues to be widely debated, with interpretation complicated by different trial populations, methods of assessing risk, as well as by differences in timing and dose of radiation. [24]

3. Targeted small molecule inhibitors

Targeted small molecule inhibitors are drugs that target the cancer's specific proteins that contribute to cancer growth and survival. This type of treatment blocks the growth and spread of cancer cells while limiting damage to healthy cells. Targeted small molecule inhibitors can be divided in two main classes: inhibitors of BCR signaling and Bcl-2 family inhibitors. [25]

3.1. Inhibitors of BCR signaling

BCR signaling plays a crucial part in the development of B cell malignancies. Critical components of this signaling pathway include Bruton tyrosine kinase (BTK), phosphatidylinositol-3 kinase δ (PI3Kδ), and spleen tyrosine kinase (SYK). (Figure 1) Small molecule inhibitors have been designed to block these kinases in the BCR pathway.

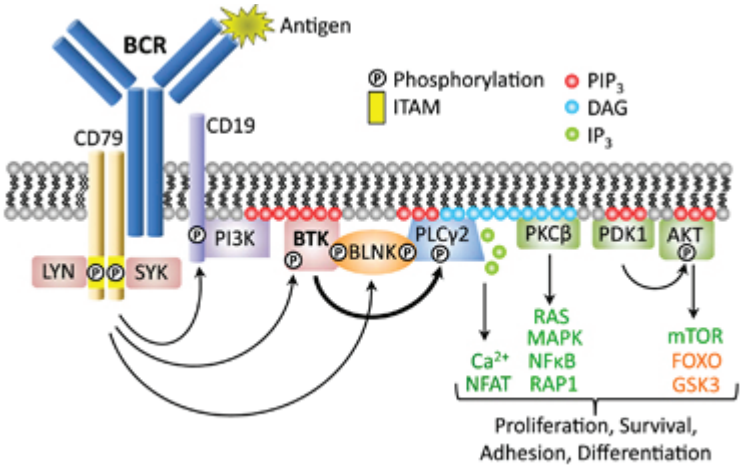


Figure 1. B-cell antigen receptor signaling in CLL.

The schema shows the major signaling pathways activated after BCR activation and linking the BCR to biologic responses. Adapted from *Spaafgen et al. 2004*

3.1.1. Bruton tyrosine kinase inhibitors

BTK is involved in multiple signal transduction pathways regulating survival, activation, proliferation and differentiation of B-lineage lymphoid cells. Both

covalent and noncovalent BTK inhibitors are being developed as therapeutic agents for various indications. Inhibition of BTK has been associated with reduction in cell migration, proliferation and survival. [26] Ibrutinib, the most clinically advanced small molecule inhibitor of BTK, has demonstrated impressive tolerability and activity in a range of B-cell lymphomas. It is an orally available small molecule that acts as an irreversible inhibitor of BTK by binding to the cysteine-481 amino acid, near the active site. Ibrutinib is currently approved by the US Food and Drug Administration (FDA) for untreated and relapsed CLL, relapsed mantle cell lymphoma (MCL), Waldenstrom's macroglobulinemia, and marginal zone lymphoma (MZL). [27] In addition to ibrutinib, several other BTK inhibitors are in various stages of development, such as LFM-A13, dasatinib, ONO-4059, CC-292 and acalabrutinib (ACP-196). [27]

3.1.2. Phosphatidylinositol 3-kinase inhibitors

The BCR pathway also signals through PI3K, which activates AKT and mTOR to promote proliferation and survival of malignant cells. The deregulation of this pathway has been associated with several types of NHL. [28,29] The isoform PI3Kδ is highly expressed in CLL cells. Idelalisib is a potent oral inhibitor of PI3Kδ, currently approved for use in refractory FL and CLL. [30,31] It is used as a single agent, or with combination with and chemotherapeutic regimens or monoclonal antibodies. Second generation PI3K inhibitors in development include: duvelisib, TGR-1202, and copanlisib. [25]

3.1.3. Spleen tyrosine kinase

The tyrosine kinase SYK is critical for normal B-cell development and proliferation and recent studies implicate this protein in NHL pathogenesis. SYK inhibition by targeted small molecules inhibitors results in decreased mTOR activity in MCL, Burkitt's lymphoma and CLL. [32] Fostamatinib disodium, a competitive inhibitor of SYK is the first that has been moved to the clinic for the treatment of patients with relapsed chronic lymphocytic

leukemia. Several other selective inhibitors of SYK and dual SYK/JAK inhibitors are currently under development as single agents or in combination with other agents, including entospletinib, R406, R788, C-61, CG14979 and PRTO62607. [26]

3.2. Bcl-2 family inhibitors

Cell survival is regulated by a dynamic balance between proapoptotic and antiapoptotic members of the BCL2 family of proteins. Inhibition of pro-survival signaling is a promising therapeutic target for hematological malignancies with BCL2 dependence such as CLL. Venetoclax (ABT-199) is a selective BCL2 inhibitor that exerts its effect independent of BCR signaling (p53 independent apoptosis). Clinical activity of venetoclax has been demonstrated in patients with relapsed CLL and MCL. [33,34] Venetoclax has been approved in certain forms of CLL.

4. Immunotherapy

Immunotherapy is a treatment that re-awakens the immune system so it can fight cancer, infection, and other diseases. Some types of immunotherapy only target certain cells of the immune system, while others affect the immune system in a general way. Several different types of immunotherapies have been developed for B-cell malignancies: monoclonal antibodies, cytokines, chimeric antigen receptor (CAR) T-cell therapy, and vaccines.

4.1. Monoclonal antibody therapy

Monoclonal antibody therapy has also been referred to as passive immunotherapy as opposed to adoptive immunotherapy (such as that represented by infusion of immune cells).

Rituximab was the first monoclonal antibody (mAb) developed for therapeutic purposes. It targets the CD20 molecule, a membrane protein found on the surface of all mature B-cells that typically has a constitutive and constant

expression and therefore provides an excellent therapeutic target. Rituximab represented a major therapeutic advance in this disease, in particular for the treatment of indolent and aggressive forms of B-cell NHL and CLL. [35] Following rituximab, several other anti-CD20mAbs have been developed and FDA approved for the treatment of B-cell malignancies, including tositumomab, ofatumomab, ibbritumomab and obinutuzumab. [36] Anti-CD20 antibodies for the treatment of B-cell malignancies are discussed in details in chapter III.

In addition to anti-CD20 mAbs, other antibodies directed against other surface antigens such as CD22 (epratuzumab), CD23 (lumiliximab) and anti-CD80 (galiximab) have been developed. [37–40]

Another highly attractive approach is the application of the bispecific T-cell engaging (BiTE) antibody blinatumomab which targets both CD19 and CD3 antigens. Although blinatumomab is currently used only for the treatment of B-precursor acute lymphoblastic leukemia, promising early results were also obtained in B-cell lymphomas, where blinatumomab was used as a single agent. [41,42]

4.2. Adoptive T cell therapies

Adoptive T-cell immunotherapy offers a novel therapeutic approach that aims to exploit the graft versus leukemia effect. The adoptive T-cell immunotherapy technique arises from use of chimeric antigen receptor (CAR) T cells. CAR T cells are autologous T lymphocytes genetically modified to express CAR constructs that include one or more co-stimulatory molecules, such as CD28, OX40 or CD19. [43,44] Following ex-vivo expansion and manipulation, CAR T-cells are infused back into the patient. Remarkable clinical results were obtained in B-cell malignancies, in particular DLBCL and CLL treated with CAR T cells with specificity for CD19. [45] Infusions of autologous anti-CD19 CAR T cells have shown a complete eradication of blood and bone marrow B cells for prolonged periods of time, demonstrating their powerful biological activity. [46]

4.3. Cytokines

Significant research efforts have explored the usage of cytokines as immunomodulators to enhance host immune response against cancer. The most commonly used cytokines are the interferons (IFN). Interferon alpha (IFN-α), is a cytokine produced by leukocytes to help the immune system fight infections. Recombinant IFN-α has shown cytotoxic activity against B-cell malignancies by activating IFN-AR signaling in B cells and thus inducing apoptosis, inhibiting proliferation and cell cycle progression. [47–49] Although IFN-α demonstrates great anti -tumor activity, its significant systemic toxicities result in a very narrow therapeutic index.

Chapter III:
Anti-CD20 monoclonal antibodies

Over the past decades, the advances in field of oncology have provided new insights into the tumor biology, growth and resistance pathways. Recognition of novel targets on the cancer cell has enabled development of monoclonal antibodies as tools to attack those targets. Monoclonal antibody therapy with the anti-CD20 mAbs represents one of the most important advances in the treatment of lymphoproliferative disorders.

1. Structure and development of monoclonal antibodies

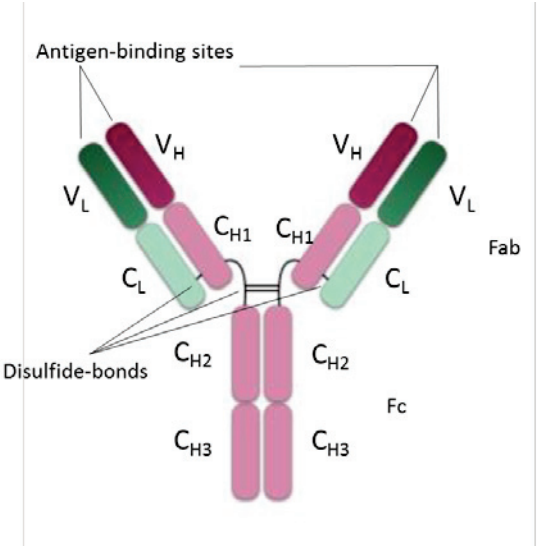
In 1975, Kohler and Milstein inaugurated a new era in antibody research with their discovery of hybridoma technology capable of producing antibodies directed toward a single antigen. [50] Hybridoma cells were produced from a human cancer cell fused to a mouse B cell, making the mouse B cell immortal and capable of producing antibody directed against a single antigen. Further improvement in antibody engineering technology has allowed the construction of various antibodies, evolving from murine, via chimeric and humanized, to fully human mAbs, which are better tolerated, with decreased immunogenicity.

An antibody is a Y-shaped protein, belonging to the immunoglobulin superfamily. It is composed of two identical heavy class (H) chains and two identical light (L) chains. Both heavy chains are linked to each other and to one light chain by disulfide bonds. (Figure 2) Each heavy chain contains an N-terminal variable region (VH), three constant regions (CH1, CH2, CH3) and a flexible "hinge" region between CH1 and CH2. Similarly, the light chains are composed of an N-terminal variable region (VL) and one constant region (CL). The pair of VH and VL regions forms the antigen-binding site. The upper part of the antibody composed of the light chain with VH and CH1 of the heavy

chain forms the Fab arm (fragment antigen binding). The rest of the antibody fragment forms the Fc fragment (fragment crystallizable) involved in immune effectors function. Antibodies are grouped into five classes according to their constant region: IgG, IgM, IgA, IgD and IgE. The classes of antibody differ not only in their constant region but also in activity. IgG is the most stable one and the most abundant in human serum, and therefore most frequently used in immunotherapy.

Figure 2. Structure of an antibody.

Antibodies are Y-shaped, flexible molecules consisting of two heavy (pink) and two light (green) chains linked together by disulfide bonds. The light and heavy chains are composed of constant (C L , C H1 , C H2 , C H3) and variable (V L , H L) regions.



Monoclonal antibodies are antibodies that are identical and specific for the same epitope. In early studies, the therapeutic monoclonal antibodies were of murine nature. Mouse antibodies, being different from human antibodies and therefore immunogenic, produced an inflammatory reaction when injected into humans, resulting in immune reactions and the production of neutralizing antibodies (Human Anti Murine Antibodies) which would limit the clinic benefit of therapeutic mAbs. In order to overcome these difficulties various different kinds of approaches have been used, leading to the production of chimeric (partly mouse and partly human) (suffix -ximab), humanized (suffix -zumab) and fully humanized antibodies (suffix - umab).

2. Mechanisms of action

A variety of mechanisms are thought to play important roles in mediating the observed anti-tumor effects of mAb. These include direct action by binding to surface antigen that leads to cell death, blocking an activation signal that is necessary for continued cell growth, as well as indirect effect via the recruitment of immune system effectors: antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC).

Based on their mechanism of action, anti CD20 mAbs can be divided into two distinct subtypes, type I and II. Type I antibodies display a remarkable ability to activate complement and induce CDC through enhanced recruitment of C1q, while type II are relatively ineffective in CDC. On the other hand, type II antibodies mediate superior direct killing of target cells, while type I antibodies are poorly efficient in inducing direct cell death. Both type I and II mAbs appear to demonstrate efficient antibody-dependent cellular phagocytosis (ADCP) and ADCC. Finally, type I but not type II mAbs redistribute CD20 into the lipid rafts. [51] (Figure 3)

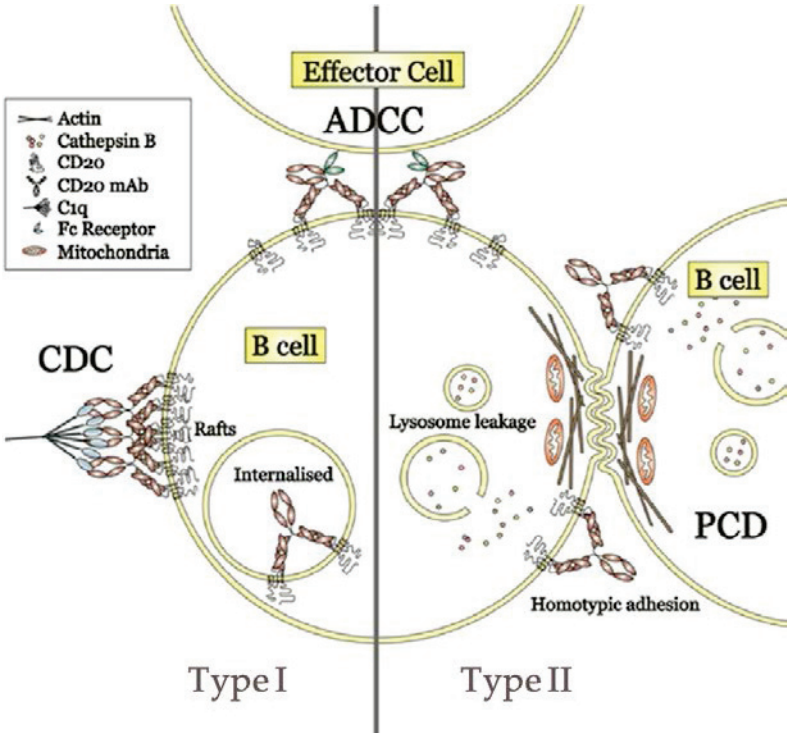


Figure 3. Different mechanisms of action of type I and II anti-CD20 mAbs.

Type I mAbs are able to engage CDC and ADCC and cause modulation in certain B-cell malignancies but do not elicit efficient direct cell death, whereas type II mAbs can mediate direct cell death through a lysosomal pathway and engage ADCC but do not promote CDC. Adapted from *Beers et al.* 2010. [51]

2.1. Direct cell death induction

The Fab portion of monoclonal antibodies have antigen binding activity that can therefore either neutralize the antigen or activate signaling pathways within the target cell. Indeed, several studies demonstrate that some mAbs induce tumor cell death by activating proapoptotic signaling cascade that leads to a death signal or by enhancing the susceptibility of target cells to cytotoxic therapy through the modulation of anti-apoptotic pathways. [52,53] Apoptosis is controlled by both positive and negative factors. Accessory proapoptotic factors, such as Bax, Bad, and Smac or anti-apoptotic factors such as Bcl-2, Mcl-1, Bcl-XL and inhibitor of apoptosis (IAPs) participate in the regulation of the apoptotic process at key steps. [54] Evidence demonstrates that these factors are likely to be responsible for the extremes in susceptibility of tumors to conventional chemo- or radiotherapy. Several studies have pointed out that monoclonal antibodies can alter the apoptotic signaling pathway in target cells and thus act as chemosensitizing agents of drug–resistant cells. [55,56]

The strength of this direct cytotoxic effect varies considerably depending on the mAb, the target antigen and the target cell. Nevertheless, direct cell death induction can play an important role in the mechanisms of action of certain mAbs.

2.2. Complement dependent cytotoxicity

Monoclonal antibodies can also mediate cytotoxicity via recruitment of complement factors in the serum, which is known as complement dependent cytotoxicity. Antibodies bound to a cell surface activate complement through the classical pathway when the complement component C1 recognizes the Fc portion of mAb and becomes activated, recruits and activates other complement factor. This results in a formation of the membrane attach complex (MAC), which creates pores on the membrane of the target cells leading to cell death. [57] For the induction of strong CDC activity, various biological and structural features of antigen molecules are required, such as

relatively high expression, the presence of small or folded extracellular portions, or epitopes that retarget antigens into lipid rafts.

2.3. Antibody-dependent cellular cytotoxicity

Monoclonal antibodies can induce ADCC mediated by a variety of immune effector cells including natural killer (NK) cells, granulocytes and macrophages. These processes require that the Fc of the antibody bound to the target cell bind to Fc γ receptor (FcγRs) on the effector cells triggering immune cell activation leading to signaling cascades that result in the release of cytotoxic granules (containing perforin and granzyme B) and thus death of the target cell. [58] The effector functions have been considered to play a key role in the clinical efficacy of therapeutic mAbs. FcγRIIIa (CD16A), is known to be a major triggering receptor of ADCC in NK cells and may thus be one of the major critical mechanisms. This ADCC mechanism of therapeutic mAbs underlines the importance of the presence of effector immune cells bearing FcγR for their antitumor activity. [59]

2.4. Antibody-dependent cellular phagocytosis

Another mechanism of action of mAbs involves phagocytosis of antibody coated tumor cells by macrophages expressing FcγRII (CD32). This mechanism, called antibody-dependent cellular phagocytosis, has been demonstrated to play an important role in tumor cell depletion, both *in vitro* and in *vivo*. [60,61] Also, clinical studies have shown that polymorphisms of FcγRII could predict clinical outcome of several different mAbs therapy. [62–64]

Altogether, mAbs induce specific killing of target tumor cell, either by directly triggering signaling cascades leading to cell death or through immune effector cells. Even though each mAb appears to have a predominant mode of action, the antitumor activity of mAbs *in vivo* is likely result of several or all mechanisms of action. In the clinic, mAbs are often used in combination with other approaches, such as chemotherapy, radiotherapy or immune

modulators. [65] In addition, different strategies have been developed to enhance their therapeutic potential, such as drug-conjugated antibodies, glycoengineering and bispecific antibodies. [66]

3. CD20, a target for mAbs

The CD20 antigen is a transmembrane phosphoprotein of the tetraspanin superfamily that is exclusively expressed on the B-cell lineage. [67] Its expression begins at the pre-B stage of B-cell ontogeny and continues until the mature stage, but is absent on plasma cells and hematopoietic stem cells. CD20 is also highly expressed on malignant B cells in NHL and CLL, making these malignancies susceptible to immunotherapeutic strategies targeting CD20. Additionally, it does not circulate freely in the blood that could competitively inhibit MAb binding to lymphoma cells. Also, it is not normally shed from the surface of the B-cell upon antibody binding, nor is internalized or, subsequently, down-regulated after antibody binding. All of these characteristics make the CD20 antigen an appealing target for monoclonal antibody therapy. [51,68–70]

Nevertheless, despite the therapeutic success of anti-CD20 antibodies, very little is known about the biology of CD20. It has no known ligand, and CD20-knockout mice do not show a prominent phenotype. [71,72] Therefore, much of the current understanding about this molecule comes from prediction based upon the gene and protein structure. The reported oligomeric and tetraspan structure of CD20 has led to suggestions that CD20 may form a membrane channel and play a role in the regulation of ion flux. The suggestion was supported by the observation that CD20 negative cells transfected with CD20 resulted in increase in the basal level of intracellular Ca^{2+}. [73,74] Additionally, interesting insight has come from work showing that CD20 is resident in lipid raft domains of the plasma membrane where it is rapidly reorganized when cross-linked with mAb. [75] These various studies point to the biological function of CD20 as a possible regulator of calcium signaling.

4. Rituximab

Reff *et al.*, in a landmark paper in 1994, reported on the construction of the chimeric anti-CD20 monoclonal antibody rituximab. Rituximab is a highly specific mouse/human chimeric antibody engineered by grafting the variable regions targeting the CD20 antigen from a murine anti-CD20 antibody (2B8) onto human constant regions. [76] Since its FDA approval in 1997, rituximab has revolutionized the therapeutic approach for patients with wide range of B cell malignancies. [77]

Rituximab is a type I anti-CD20 monoclonal antibody, which stabilizes CD20 on lipid rafts, promotes antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity activities. In the absence of immune effector mechanisms, rituximab has a modest efficacy in inducing direct cell death. Several studies suggest that rituximab sensitizes malignant B cells to chemotherapy, mainly through disruption of anti-apoptotic pathways. [55,56] Thus, synergy with chemotherapeutics is an important mechanism of action of rituximab.

Rituximab displayed an impressive overall response rate (ORR) of 48% in the pivotal study performed in relapsed anti-CD20 patients with a history of indolent lymphoma. [78] Today, rituximab is approved for treatment of relapsed and refractory indolent lymphomas as single-agent therapy and as initial therapy in combination with standard chemotherapy regimens. In patients with DLBCL, it is approved for use as initial therapy with CHOP or other anthracycline-based chemotherapy. Rituximab is also approved for use with chemotherapy in previously treated and untreated patients with CLL. [35,79] It is generally well tolerated; however, as a chimeric antibody, it may precipitate cytokine mediated reactions. [80]

5. Obinutuzumab (GA101)

Obinutuzumab (GA101) is a humanized, glycoengineered anti-CD20 monoclonal antibody with enhanced induction of direct cell death and antibody-dependent cell-mediated cytotoxicity. (Figure 4) [81,82] GA101 was designed as a type II antibody. Type II CD20 binding was achieved by the introduction of sequence variations into the variable part of the antibody. The most potent induction of direct cell death was achieved by a variation in the elbow hinge region of the antibody (valine instead of leucine). [81] Additionally, GA101 has a glycoengineered Fc segment leading to a reduced fucose content, thereby increased affinity for binding affinity to FcγRII and enhanced antibody-dependent cell-mediated cytotoxicity. These glycoengineered modifications resulted in significantly reduced CDC activity. [83] Nevertheless, even in absence of CDC; *in vitro* and *in vivo* studies, have showed that GA101 has a significant superior antitumor activity in comparison to rituximab.

In 2013, FDA approved obinutuzumab in combination with chlorambucil for the treatment of patients with previously untreated chronic lymphocytic leukemia. [84] This approval was based on demonstration of an improvement in progression-free survival (PFS) in a randomized phase III clinical trial that compared obinutuzumab in combination with chlorambucil with chlorambucil alone in patients with previously untreated CD20-positive CLL. This study also compared rituximab in combination with chlorambucil with obinutuzumab in combination with chlorambucil. 28.7% of patients who received obinutuzumab in combination with chlorambucil achieved a complete response (CR). [85] In addition, obinutuzumab in combination with chlorambucil doubled the PFS of patients with newly diagnosed CLL.

In 2016, FDA approved obinutuzumab for use in combination with bendamustine followed by obinutuzumab monotherapy for the treatment of patients with relapsed or rituximab refractory follicular lymphoma. [86] This new approval was based on demonstration of an improvement in PFS in a randomized, open-label, multicenter trial in patients with FL who had no response to or had progressed after a rituximab-containing regimen. [87]

Obinutuzumab has also been investigated as monotherapy in patients with relapsed/refractory indolent and aggressive NHL and in CLL of B-cell origin. Monotherapy with obinutuzumab for B cell malignancies showed great efficacy and acceptable tolerability. [88–91] Following the promising activity of obinutuzumab in phase I and II single-agent studies, a number of studies have been carried out in the combination therapy setting, such as obinutuzumab with CHOP, or with fludarabine and cyclophosphamide in patients with relapsed/refractory FL. [92] Several ongoing trials are also evaluating obinutuzumab in first-line CLL. In a phase III study, the efficacy and safety of a combined regimen of obinutuzumab and venetoclax is compared to obinutuzumab plus chlorambucil in patients with CLL. [93] Another study is evaluating the combination of obinutuzumab and ibrutinib, compared with obinutuzumab plus chlorambucil in patients with CLL or small lymphocytic leukemia. [94]

These further studies will help to confirm treatment benefits with obinutuzumab in patients with B-cell malignancies. Nevertheless, current evidence indicates that obinutuzumab is a useful option for patients who relapsed after or are refractory to a rituximab-containing regimen.

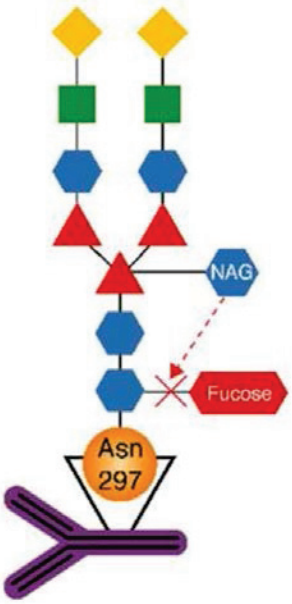


Figure 4. Glycoengineered structure of GA101

GA101 was glycoengineering by defucosylation of immunoglobulin G oligosaccharides in the Fc region of the antibody. In Chinese hamster ovary producer cells, N-acetylglucosamine (NAG) is assembled into oligosaccharides, which sterically prevents the addition of fucose to the carbohydrate attached to asparagine (Asn) 297. Adapted from *Tobinai et al.* 2017. [82]

6. Ofatumumab

Ofatumumab is a completely human type I anti-CD20 mAb that binds a different epitope than that recognized by rituximab. Compared to rituximab, it targets CD20 with greater avidity, causing similar ADCC but stronger CDC. [95,96]

In 2009, ofatumumab was approved in the USA and EU for fludarabine- and alemtuzumab-refractory CLL patients and for fludarabine refractory patients with bulky disease, on the basis of demonstration of durable tumor shrinkage. [97] Additionally, in 2016, based on the results from the phase III COMPLEMENT 2 study, ofatumumab was also approved in combination with fludarabine and cyclophosphamide for the treatment of patients with relapsed chronic lymphocytic leukemia. [98]

7. Ibritumomab tiuxetan

Ibritumomab tiuxetan or Y^{90}–Ibritumomab, is a murine anti-CD20 mAb which targets the same epitope on the CD20 antigen as rituximab. Tiuxetan, a second-generation chelator, is covalently bound to ibritumomab, allowing for formation of a stable radioimmunoconjugate by chelating Y-90. [99,100]

Ibritumomab tiuxetan was the first radio-immunotherapy drug approved by the FDA in 2002 to treat cancer. It was approved for the treatment of patients with relapsed or refractory low‑grade follicular or $CD20^{+}$ transformed B cell NHL and rituximab refractory FL. [101]

8. Tositumomab / I^{131} Tositumomab

Tositumomab is a murine IgG2 mAb, targeted against CD20. It can be labeled with iodine-131 to yield I^{131}-tositumomab. Upon binding to CD20, tositumomab induces ADCC; CDC and direct apoptosis. In addition, when radiolabeled, the antibody exploits the tumour's sensitivity to ionising radiation by direct targeting of the malignant cells. [102]

In 2003, FDA approved tositumomab and I^{131} tositumomab for the treatment of patients with CD20+ follicular lymphoma, with and without transformation, whose disease is refractory to rituximab and has relapsed following chemotherapy. However, as of February 2014, tositumomab and iodine I^{131} tositumomab have been discontinued by the manufacturer and are no longer available. [103]

9. Other anti-CD mAbs in clinical trials

9.1. Ocrelizumab

Ocrelizumab is a humanized type I anti-CD20 mAb, with superior binding affinity for the low-affinity variants of the FcγRIIIa receptor. In, addition, it showed higher ADCC and lower CDC activity as compared to rituximab. [104] Currently, this mAb has been evaluated through a phase I/II study in patients with relapsed/ rituximab refractory FL. [105]

9.2. Veltuzumab

Veltuzumab is another humanized type I anti-CD20 mAb identical to rituximab with single amino acid substitution (Asp101 instead of Asn101) within the variable heavy chain. This modification results in reduced off-rate and a superior CDC activity in comparison to rituximab. It also showed anti-proliferative, apoptotic and ADCC effects *in vitro* similar to rituximab. [106] Veltuzumab is currently evaluated in a phase I/II study in patients with CD20 + NHL and CLL. [107]

9.3. Ocaratuzumab

Ocaratuzumab (AME-133v) is an engineered humanized type I anti-CD20 mAb with enhanced Fc affinity fo FcγRIIIa. In *in vitro* assays, Ocaratuzumab showed stronger binding affinity with CD20 and a higher avidity to the low affinity variants of FcγRIIIa receptor, and thereby improving killing of NHL tumor cells as compared to rituximab. [108] It is currently being evaluated in a

Phase I/II dose escalation study in patients with relapsed/refractory follicular B-cell NHL. [109]

9.4. RhuMAb

RhuMAb (PRO131921) is a humanized anti-CD20 mAb with an engineered Fc region that provides greater binding to the low-affinity variant of FcγRIIIa. The higher binding affinity showed an improved ADCC activity as compared to rituximab. [104] Phase I/II clinical studies demonstrated better anti-tumor efficacy in patients with relapsed and /or refractory indolent lymphoma who failed rituximab containing regimens. [110] However its clinical development has been recently terminated due to safety issues of escalating doses of Pro13192 in patients with NHL and CLL.

9.5. Ublituximab

Ublituximab (EMAB-6) is another chimeric glyco-engineered IgG1 mAb with an enhanced Fcγ RIII affinity, due to a low fucose contents in its Fc region. [111] Ublituximab has similar CDC activity and direct cell death induction, whereas ADCC response rate is greater than rituximab. Phase I trials of single agent ublituximab in relapsed/refractory CLL reported significant response rates with rapid and sustained lymphocyte depletion and a manageable safety profile. [112] An ongoing phase II trial is evaluating ublituximab in combination with ibrutinib in patients with relapsed or refractory CLL and MCL. [113]

10. Resistance

In clinical applications, the efficacy of anti-CD20 mAbs seems to be decline after a period of months of treatments due to therapeutic resistance. [114] The mechanisms for this therapeutic resistance are not clearly understood. The possible mechanisms of this resistance of B-cell NHLs against anti-CD20 mAbs therapy may be diverse: (I) Protection of the tumor cells from mAbs mediated depletion of B-cell lymphoma by ADCC, CDC and apoptotic stimulation [115–117] (II) Inadequate binding of mAbs to the CD20 molecule and (III) Low or

reduced levels of CD20 antigens on cells surface or acquired mutations in CD20. [118,119]

Chapter IV
Early Growth Response -1

… a gene with as many names as biological functions.

Although most researchers in biology tend to focus on very specific issues and questions regarding one gene or pathway, sometimes we face situations in which nature presents us with a remarkable example of a gene with multiple functions.

1. Immediate early genes

Immediate early genes (IEGs) are a class of genes that are rapidly and transiently activated by a variety of cellular stimuli and do not require *de novo* protein synthesis for their expression. [120,121] They represent a standing response mechanism that is activated at the transcription level in the first round of response to stimuli. Thus IEGs have been called the "gateway to the genomic response". In their role as "gateways to genomic response", many IEG products are transcription factors or other types of DNA-binding proteins. They play pivotal roles in a wide range of biological responses, including neuronal survival and plasticity, cardiac stress response, innate and adaptive immune responses, glucose metabolism, and oncogenic transformation. [121]

About 40 cellular IEGs have been identified so far, among which the early growth response (EGR) family genes, including egr-1, -2, -3 and -4. The early growth response -1 (EGR-1) is in the main focus of the following chapter.

2. EGR family of transcription factors

Early Growth Response genes constitute a family of zinc finger transcription factors which are induced under a vast array of signals on a variety of cell types, such as fibroblasts, B and T cells. Several laboratories searching for genes whose expression was induced by growth factors first identified members of the EGR family two decades ago. As a result, the four members of this family were given multiple individual names. More recently, the growing emphasis on understanding their function as a family has fostered acceptance of the EGR-family nomenclature. A defining feature of the EGR family is a highly conserved DNA-binding domain composed of three Cys2–His2 type zinc-finger motifs. The zinc fingers of these transcription factors showed extensive conservation along the DNA-binding domains, displaying a 90% homology, which suggests common DNA target sequences among the members of this family. However other sequences located at the 3′ region are quite different, which marks the relevance of the structure in relation to the functionality of each transcription factor. [122–124] (Figure 5) Moreover, the unique functions of each member are revealed by the specific phenotypes in knockout mice.

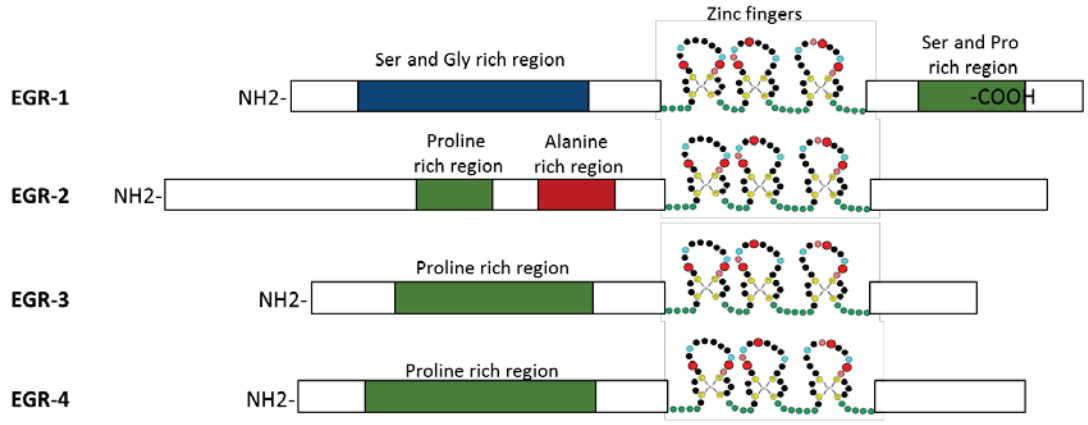


Figure 5. Structure of the EGR transcription-factor family

3. EGR-1 - a transcription factor in the crossfire of signal transduction cascades

EGR-is a nuclear phosphoprotein that was first identified based on its early induction by mitogens and differentiation factors. It is also known as NGFI-A, Zif268, krox-24 and T1S8. [125–128] EGR-1 contains a highly conserved DNA-binding domain, composed of three zinc fingers that bind to the GC-rich consensus sequence GCG(G/T)GGGCG. Unlike other transcriptional factors, EGR-1 is found to activate transcription by binding to DNA as a monomer. [122,129] In addition to the highly conserved DNA binding domain, EGR-1 also contains a nuclear localization signal, two activator domains, and one repressor domain.

The basal EGR-1 level of expression is low in most tissues and is rapidly induced in response to a broad variety of molecular and environmental stimuli including growth factors, cytokines, shear stress, oxygen deprivation and mechanical injury. Cloning of the EGR-1 promoter has revealed the presence of several response elements, including, several serum response elements (SREs), an AP-1 binding site, several cAMP regulatory elements (CREs) and Sp1 consensus sequences. [130,131] Most often, increased transcription of EGR-1 is mediated by the MAP-K families, such as ERK1/2, JNK and p38MAP-K, or by an alternative pathway NF-κB. [132–134] Additionally, the up-regulation of EGR-1 expression could be a result of receptor operated Ca^{2+} channel stimulation and a consequent elevation of intracellular calcium content. [135] (Figure 6) EGR-1 also binds to its own promoter and represses transcription, thereby initiating a negative feedback loop soon after activation. [136]

In response to distinct cellular stimuli, EGR-1 is found to regulate the transcription of a remarkable spectrum of genes involved in different cellular pathways. For example, EGR-1 regulates the expression of insulin-like growth factor-II (IGF-II), platelet derived growth factor-A (PDGF-A) and -B (PDGF-B) genes, which are known to be involved in cell proliferation [137,138]; Bcl-2, fibronectin, and nuclear factor-B, which are associated with survival and cell differentiation ; as well as p53, PTEN, and tumor necrosis factor-alpha (TNFα)

which are involved in apoptosis. [139–141] Additional relevant targets are VEGF and tissue factor, which are associated with tumor progression, and TGFβ1 which induces growth inhibition in a cell type dependent manner. [139,142] EGR-1 regulates these key genes in a well-coordinated network, acting as a central hub in many cellular pathways.

Egr1-deficient mice develop a fairly normal phenotype; however, the females are infertile due to a defect in luteinizing hormone-beta production. [143]

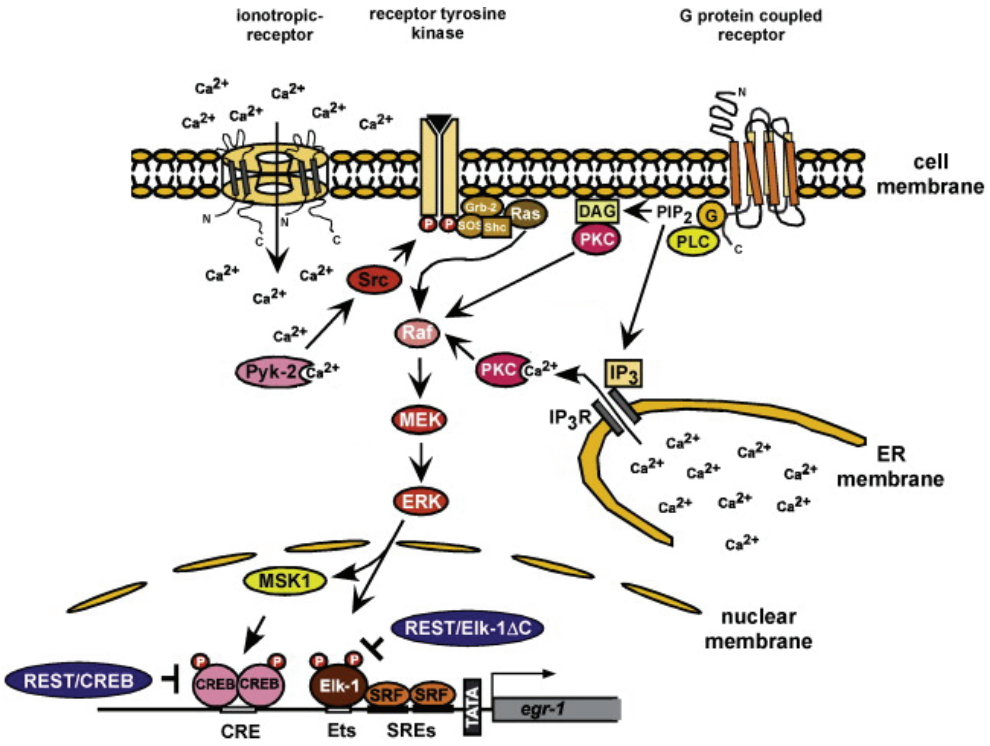


Figure 6. Signaling pathways inducing Egr-1 expression.

Signaling transduction cascades participating in the regulation of EGR-1 expression. Stimulation of G-protein-coupled receptors leads to the activation of phospholipase C (PLC), the generation of IP3 and the release of Ca^{2+} ions into the cytosol via stimulation of ionotropic IP3 receptors of the endoplasmic reticulum. Stimulation of ionotropic receptors or activation of L-type Ca^{2+} channels increases the cytosolic Ca^{2+} concentration via an influx of Ca^{2+} ions from outside. Elevation of the intracellular Ca^{2+} concentration may induce a transactivation of the EGF receptor or an activation of PKC that may involve cytoplasmic tyrosine kinases such as Pyk-2. Protein kinase C directly or indirectly regulates Raf activity. Either transactivation of the EGF receptor or activation of PKC stimulates the ERK signaling pathway. As a result of ERK activation CREB is phosphorylated and activated in the nucleus. Adapted from *Thiel et al., 2010.*

3.1. Post-translational modification of EGR-1

EGR-1 activity and stability are regulated by post-translational modifications. Acetylation, mediated by the transcriptional co-activator p300/CBP, stabilizes EGR-1 and may promote survival, whereas phosphorylation in response to stress may favor cell death. [144] Sumoylation is mediated by tumor suppressor p19ARF and directs EGR-1 to the nucleus. [145] The short-lived EGR-1 protein is ubiquitinated on multiple sites and degraded by the proteasome. [146] (Figure 7) The full spectrum of post-translational modifications regulating EGR-1 activity is far from being fully deciphered.

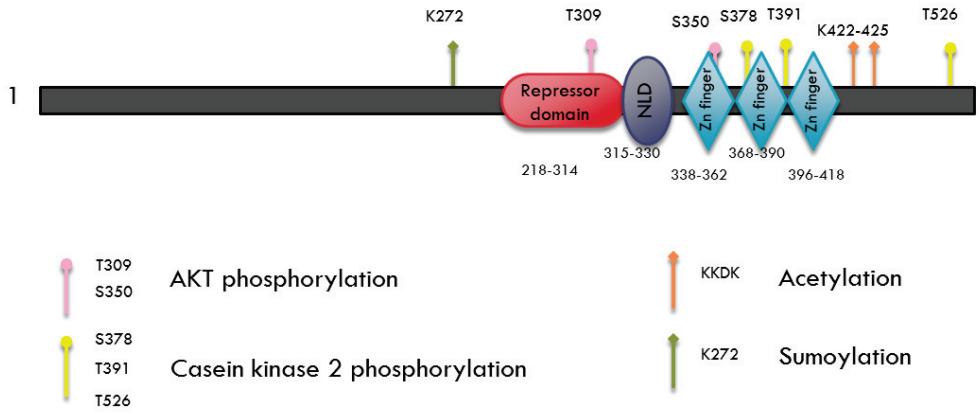


Figure 7. Structure and post-translational modification of EGR-1

EGR1 contains a highly conserved DNA-binding domain composed of three Cys2-His2 type zinc-fingers; a nuclear localization domain (NLD) and a repressor domain through which EGR1 binds to regulatory proteins NAB-1 and NAB2. Post-translational modifications include phosphorylation, acetylation, ubiquitination and sumoylation

3.2. EGR-1 and cancer

EGR-1 has several essential biological roles. It is known to be involved in the regulation of cell proliferation, but it can also induce cell death by activating apoptotic pathways. This same phenomenon could also be observed in cancer cells, where EGR-1 could behave as tumor suppressor gene or have a pro-oncogenic activity.

3.2.1. EGR-1 as a tumor suppressor gene

Analysis of certain human tumor cells and tissues indicate that EGR-1 exhibits prominent tumor suppressor function. [129,147–149] Many human tumor cell lines express little or no EGR-1 in contrast to their normal counterparts. Furthermore, EGR-1 is decreased or undetectable in small cell lung tumors, human breast tumors, and human gliomas. [149–152] The re-expression of EGR-1 in human tumor cells results in growth suppression and apoptosis, confirming its role in cell growth arrest. [147,152]

EGR-1 exerts its antitumoral effect through direct regulation of multiple tumor suppressors such as p53, transforming growth factor beta 1 (TGFβ1), phosphatase and tensin homolog (PTEN), and fibronectin. [139] The promoter of the human TGFβ1 gene contains at least two EGR1-binding sites, both of which can bind EGR1 leading to the activation of transcription. The significance of this interaction in human tumor cells has been shown by re-expression of EGR-1 in in human HT1080 fibrosarcoma cells, which do not normally express EGR-1. The re-expressing EGR-1 in HT-1080 has in synthetization and secretion of precursor TGFβ1 that becomes activated and acts in an autocrine manner to suppress growth. [147,153] Additional studies have documented functional TGFβ1 expression in correlation with or following expression of Egr1 in other cells types as well as *in vivo.* [154–156] Another tumor suppressor gene regulated by EGR-1 is PTEN, a proapoptotic factor that is frequently suppressed in a variety of human cancers. In fact, EGR-1 is the first transcription factors shown to be direct et functional regulator of PTEN. [157] Several studies have shown that the silencing of EGR-1 gene expression is paralleled by the absence of PTEN expression. [158,159] Another recognized tumor suppressor role of EGR-1 is as a ‘‘gatekeeper’’ of p53-dependent growth-regulatory mechanisms in replicative senescence and cell growth. [160] EGR-1 directly induces the transcription of p53 and may also bind with p53 in a cytoplasmic complex. [161] Studies of the EGR-1 knock-out (KO) in mice have revealed that EGR-1 normally controls p53 expression leading to cell cycle arrest and senescence. Primary fibroblasts isolated from EGR-1-KO embryos are characterized by rapid and immortal growth, as well as insensibility to genotoxic stress with no blockage following DNA damage.

Additionally, these cells express little or no p53 although the gene is intact. These properties can be rescued by re-expressing either EGR-1 or p53, indicating that EGR-1 is a key regulator of p53 and plays a central functional role in p53-dependent growth control *in vivo*. [148,160]

As EGR-1 regulates a network of major tumor suppressor genes, an aberration in its expression would favor transformation and survival of cancer cells. Therefore, EGR-1 could be an interesting candidate for gene therapy.

3.2.2. EGR-1 as an oncogene

Although EGR-1 regulates a functional network of tumor suppressor factors, paradoxically, it is oncogenic in prostate cancer. An increased EGR-1 expression is consistently observed in prostate cancer cells, as well as epithelial and stromal cells at tumor margins but not in lymph node metastases or healthy prostate tissue. [162] Moreover, the level of Egr1 increases with the degree of malignancy as measured by the Gleason score of the tumor, and several EGR1 target genes are described to be implicated in prostate tumor progression. [162,163] In addition, a majority of primary human prostate carcinomas have lost expression of the EGR-1 corepressor NAB2, resulting in high and unrestrained EGR1 transcriptional. [164] Nevertheless, despite EGR-1 overexpression in prostate cancer, it does not seem to be required for tumor initiation since tumors do develop in EGR-1 null transgenic mouse models of prostate cancer. However, EGR-1 appears to play a key role in tumor progression, as EGR-1 deficiency in mice delayed the progression from prostatic intra-epithelial neoplasia to invasive carcinoma. [165] The particular role of EGR1 in prostate cancer could be due to a crosstalk with the androgen receptor, with whom it physically interacts, which results in an increased proliferation. [166] EGR1 also stimulates the production of many growth factors and cytokines, leading to autocrine cell growth and to alteration of the tumor environment, and thus directly contributes to cancer progression. [167]

There are few other examples of EGR-1 overexpression in cancer, including Wilm's tumor, kidney cancer and *B-RAF* mutated melanoma. [168,169]

These observations that EGR-1 also regulates a gene program that promotes cancer progression, has thus identified a paradoxical function in cancer. Further elucidation of its mechanism of action and EGR1-related signaling networks in human tumors remain essential.

3.3. EGR-1 in B cells

EGR-1 is ubiquitously expressed at a low level in all cells. In mature B-cells, signaling through BCR results in rapidly induced EGR-1 expression through activation of the p21 ras/MAPK pathway. [170,171] Studies with EGR-1-specific antisense oligonucleotides have demonstrated that induction of EGR-1 is necessary for antigen receptor-mediated activation of B and T lymphocytes. [172,173] However, in contrast to mature B cells, it has been shown that EGR-1 gene is methylated and not induced upon BCR ligation in immature B cells. [170,172,174] Thus, the major difference observed between mature and immature B cells in response to antigen receptor-mediated signaling appears to be the variation in expression of EGR-1, suggesting that it might be important for tolerance induction in B cell. [175] Although EGR-1 seems to play an important role in B cell development, it's role in B-cell malignancies has been poorly studied. Nevertheless, it has been shown that EGR-1 expression in stromal cells in contact with lymphoma cells inhibits MMP-9 expression and consequently the growth of lymphoma cells. [176]

4. Early growth response -2

Similarly, to EGR-1, EGR-2 is also a transcription factor rapidly induced by multiple extracellular stimuli, in particular cytokines. [177] In contrast to EGR-1, which has been extensively investigated, the regulation and function of EGR-2 remains less well characterized. It shares a conserved protein structure, composed of three C2H2 zinc fingers, binding to the consensus nucleotide sequence GCGGGGGCG, to regulate expression of target genes. It is found to play an essential role in brain development, as well as in regulating the development of regulatory T cells, dendritic cells, and the differentiation of T

helper 17 cells. EGR-2 knockout mice die perinatally because of defects in hindbrain development, and therefore its function in T cell development has not been studied. RNA interference-mediated knockdown of EGR-2 in T-cell line rendered the cells less susceptible to anergy induction, whereas overexpression of EGR-2 reduced T cell activation *in vitro*, indicating that Egr-2 regulates genes involved in the suppression of T cell activation. [177,178]

5. Early growth response – 3

EGR-3 (also known as PILOT) is an immediate early growth response gene, that was first described in 1991 to be induced by mitogenic stimulation of rodent and human fibroblasts and monkey kidney epithelial cell line. [179] The expression of EGR-3 has been reported in various tissues, such as lymphocytes, muscle, endothelial cells and different brain regions. [122,180] EGR-3 has a vital role in muscle-spindle development as evidenced by EGR3-deficient mice displaying severe motor abnormalities and defects in muscle development. About 40% of EGR-3-deficient mice died perinatally, and therefore it is difficult to examine the other roles of EGR-3. [181] Nevertheless, several studies indicate that EGR-3 has a regulatory activity in T cell tolerance and thus autoimmunity. [178,182,183] Double EGR-2 and EGR-3 knock-out in mice results in lethal inflammatory autoimmune syndrome with lymphocytic infiltration in multiple organs. [182] Conversely, EGR-3 conditional knock-out mice do not develop any autoimmune symptoms, and a compensatory role for EGR-2 in T-cells is suspected. [182] The best described role for EGR-3 in $CD4^+$ T cells is the control of TGF-β1 and IL-10 production, as confirmed by the observation that EGR-3-deficient $CD4^+$ T cells produce decreased amounts of TGF-β1. [184] Therefore EGR-3, which can upregulate TGF-β1 production in T-cells, is a potential target for the treatment autoimmune diseases.

6. Early growth response - 4

EGR-4 (also known as AT133) was initially identified as a gene induced s by nerve growth factor, in the central nervous system. It is mostly expressed at

high levels in the forebrain it is identified as a novel regulator of posterior hindbrain development. [185] EGR-4 is also detected at low levels in testicular germ cells and EGR-4 null mice are characterized by a defect in germ cell maturation, leading to male infertility, indicating a critical role for EGR-4 in spermatogenesis. [186] EGR-4 remains the least explored member of the EGR family, and its biological roles are yet to be elucidated.

Chapter V
Calcium signaling

Calcium ions (Ca^{2+}) impact nearly every aspect of cellular biology. Unlike many other second-messenger molecules, Ca^{2+} is required for cell survival, yet prolonged high intracellular Ca^{2+} levels lead to cell death. A tight control of basal cytosolic Ca^{2+} concentration is essential to fine-tune Ca^{2+} dependent cell functions.

1. Calcium channels

Calcium ion channels are widespread in all cell types and regulate many fundamental physiological functions. Regulation of Ca^{2+} homeostasis involves both entry from extracellular space and release from intracellular stores. The ubiquitous second messenger Ca^{2+} regulates cell cycle control, survival, apoptosis, migration and gene expression. For each cellular function, specific spatial and temporal characteristics are required. Two major classes of channels mediate Ca^{2+} entry in response to various stimuli: voltage-gated Ca^{2+} channels and non-voltage gated calcium channels.

1.1. Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels (CaV) mediate Ca^{2+} entry in excitable cells in response to membrane depolarization. They can also contribute to the regulation of Ca^{2+} entry in non-excitable cells such as T cells and cancer cells. Cav channels are heteromultimers, composed of a pore-forming $\alpha 1$ subunit, a transmembrane complex of $\alpha 2$ and δ subunit, an intracellular β regulatory subunit and in some cases a transmembrane subunit. [187] (Figure 8) Ca^{2+} currents recorded in different cell types have diverse physiological and pharmacological properties. Based on these criteria distinct types of Ca^{2+}currents have been classified in L-, N-, P-, Q-, R-, and T-type. [188] The

different types of Ca^{2+} currents are primarily defined by different $\alpha 1$ subunits. These subunits can be divided into three structurally and functionally related families (CaV1, CaV2, and CaV3). [189] L-type Ca^{2+} currents (CaV1.1 through CaV1.4) require a strong depolarization for activation, are long lasting, and are blocked by the organic L-type Ca^{2+} channel antagonists, including dihydropyridines, phenylalkylamines, and benzothiazepines. They are the main Ca^{2+} currents recorded in muscle and endocrine cells, where they initiate contraction and secretion. N-type (CaV2.1), P/Q-type (CaV2.2), and R-type (CaV2.3), Ca^{2+} currents also require strong depolarization for activation. They are expressed primarily in neurons, where they initiate neurotransmission at most fast synapses. T-type (CaV3.1 through CaV3.3) Ca^{2+} currents are activated by weak depolarization and are transient. They are expressed in a wide variety of cell types, where they are involved in shaping the action potential and controlling patterns of repetitive firing. [188–190].

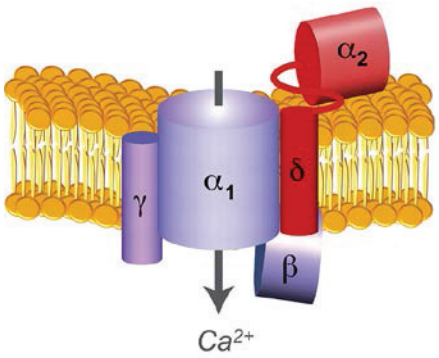


Figure 8. Molecular organization of voltage-gated Ca^{2+} channel subunits in the plasma membrane

Graphic representation of the high voltage-activated calcium channel complex consisting of the main pore forming $\alpha 1$-subunit plus ancillary, β-, γ-, and $\alpha 2$-δ-subunits. Adapted from *Khosravani et al., 2006.*

1.2. Non-voltage-gated Ca^{2+} channels

Non-voltage-gated Ca^{2+} channels are the major Ca^{2+} entry pathways in non-excitable cells. There are two main types of non-voltage-gated Ca^{2+} channels: Ca^{2+} Release Activated Ca^{2+} channel (CRAC) also known as Store Operated Channels (SOCS) and Store Independent Ca^{2+} Channels (SIC).

1.2.1. Ca^{2+} Release Activated Ca^{2+} channel

Ca^{2+} Release Activated Ca^{2+} channels are highly Ca^{2+}-selective store-operated channels expressed in T cells, mast cells, and various other tissues. CRAC

channels are composed of tetrameric assemblies of Orai 1 protein that form the channel pore and are activated by stromal interaction molecules (STIM) 1 and 2. Located in the membrane of the endoplasmic reticulum, STIM1 and STIM2 have the dual function of sensing the intraluminal Ca^{2+} concentration in the ER and to activate CRAC channels. [191–193] (Figure 9) Ca^{2+} entry through CRAC channels drives exocytosis, stimulates mitochondrial metabolism, activates gene expression, and promotes cell growth and proliferation. [194]

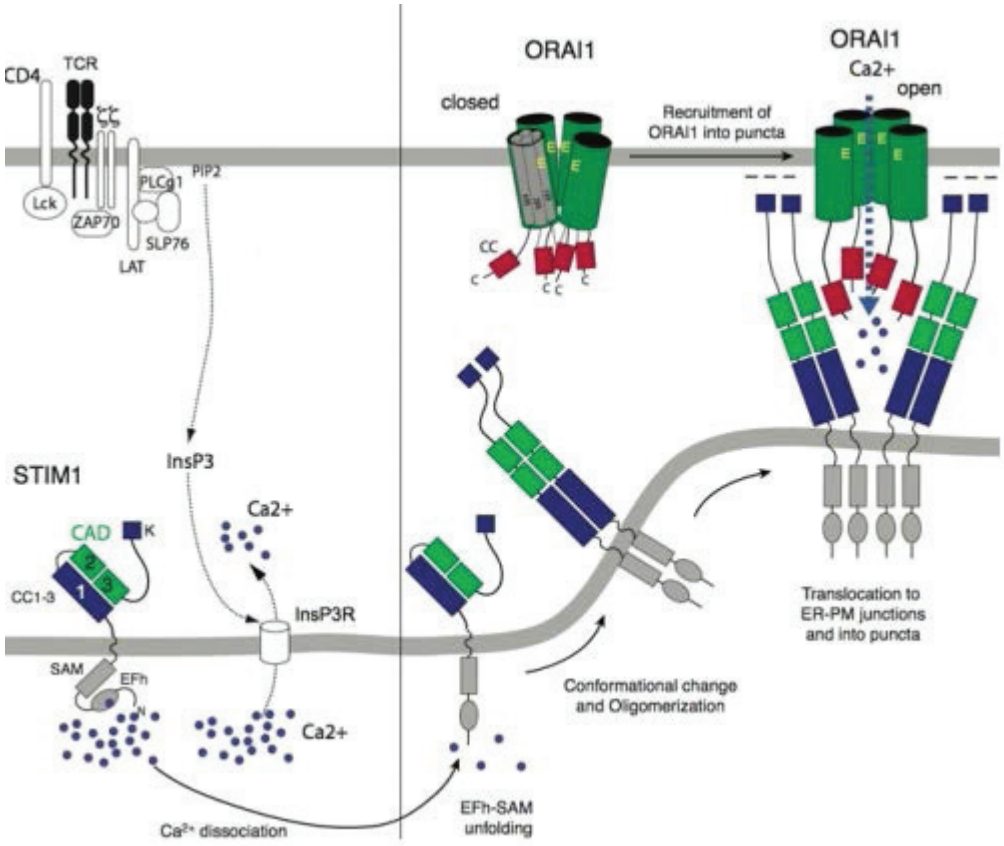


Figure 9. The molecular choreography of CRAC channel activation

In resting lymphocytes, ER Ca^{2+} stores are filled with Ca^{2+} bound to the EF hand Ca^{2+} binding domain in the N-terminus of STIM1. Antigen receptor stimulation causes the activation TCR-proximal signaling cascades and the production of InsP3, resulting in the release of Ca^{2+} from the ER through InsP3 receptors, which are non-selective ion channels. The fall in ER Ca^{2+} concentration leads to the dissociation of Ca^{2+} from the EF hand domain in STIM1, unfolding of the STIM1 N-terminus and the multimerization of STIM1 proteins. STIM1 multimers translocate to junctional ER sites in which the ER membrane is juxtaposed to the plasma membrane. STIM1 multimers form large clusters (or puncta) into which they recruit ORAI1 CRAC channels. A minimal CRAC channel activation domain in the C terminus of STIM1 is necessary and sufficient for ORAI1 binding, CRAC channel activation, and SOCE.

1.2.2. Store Independent Ca^{2+} Channels

SIC include receptor operated channels (ROC), which are directly activated in response to extracellular signals to surface receptors without intracellular signalization, and second messenger operated channels (SMOC), which are activated by an intracellular ligand generated by the activation of G protein-coupled receptor. [190] A prototype of SIC channels are arachidonate-regulated Ca^{2+} (ARC) channels, which have been shown to be activated by exogenous arachidonic acid. [195] ARC channels display biophysical properties that closely resemble those of CRAC channels but, whereas the latter are formed exclusively of Orai1 subunits, the ARC channel is formed of a combination of Orai1 and Orai3 subunits. [196] ARC channels have been shown to be present in a variety of different cell types including acinar cells of the exocrine pancreas and parotid gland. [197,198] ARCs appear to have a major role in providing the predominant mode of Ca^{2+} entry at the low agonist concentrations that induce oscillatory Ca2+ signals, where they act to modulate the frequency of these oscillations. [198,199]

2. Calcium entry

The process of calcium influx or calcium signaling can occur either by capacitive entry in response to depletion of intracellular Ca^{2+} stores or by constitutive Ca^{2+} entry independent from intracellular Ca^{2+} stores.

2.1. Capacitive Ca^{2+} entry

Capacitive Ca^{2+} entry, also called store-operated Ca^{2+} entry (SOCE), is a major mechanism for Ca^{2+} influx in non-excitable cells via the CRAC channels. Capacitive Ca^{2+} entry is triggered by the depletion of intracellular Ca^{2+} stores, in particular from the lumen of the endoplasmic reticulum (ER). [200,201] The resulting decrease of Ca^{2+} concentration in the ER is sensed by the stromal interaction molecule 1 (STIM1), which then translocates to the plasma membrane, where it interacts with the calcium channel Orai 1, leading to Ca^{2+}

influx from extracellular space to restore the Ca^{2+} concentration in ER. [202] STIM1 is a single-transmembrane domain protein that is mostly localized in the ER membrane. It has an EF-hand Ca^{2+} binding domain through which it senses the ER luminal Ca^{2+} concentration. The decreased ER Ca^{2+} content results in a profound intracellular redistribution of STIM1 from a uniform ER pattern to spatially discrete areas, called puncta. STIM-1 puncta are found in close proximity to the plasma membrane, allowing STIM1 to be translocated to the plasma membrane and interact with Orai1 in response to store depletion. [203–206] Orai1 is a tetra-spanning plasma membrane protein and functions as a highly Ca^{2+} selective channel. Orai1 opens in response to the signal conveyed by STIM1, transports Ca^{2+} selectively, and, in certain cases, directs the intracellular Ca^{2+} signal to privileged effectors. [205,207,208]

Physiologically, Ca^{2+} store depletion is controlled by various channels, among which the inositol-1,4,5-trisphosphate receptor and ryanodine receptor. [209,210] The principal activator of these channels is Ca^{2+} itself, and this process of calcium-induced calcium release is central to the mechanism of Ca^{2+} signaling. [211]

2.2. Constitutive Ca^{2+} entry

Some calcium channels are open and active under basal conditions, without n the absence of any external stimulation, thus inducing constitutive Ca^{2+} entry independently from intracellular stores (SICE). [212,213] These Ca^{2+} channels support Ca^{2+} entry into the cytosol along its electrochemical gradient across the plasma membrane, leading to an increase of cytosolic free Ca^{2+}. Various Ca^{2+} channels are involved in the constitutive Ca^{2+} influx, including voltage-gated Ca^{2+} channels, ligand-gated channels, secondary messenger-operated channels linked to G-protein-coupled receptor or receptor tyrosine kinase activation and production of secondary messengers, as well as store-operated channels. [213] Among these channels, the Transient Receptor Potential (TRP) family of cationic channels permeable to Ca^{2+} play an important role in the cytoplasmic Ca^{2+} content. [214] In response to a various range of stimuli, including binding of intra- and extracellular messengers, changes in

temperature, chemical agents, mechanical stimuli, and osmotic stress, TRP channels act directly by supporting Ca^{2+} entry through the plasma membrane, or indirectly through modulation of the membrane potential controlling the driving force for Ca^{2+} entry. [213,215,216] Along TRP channels, STIM1 and the Orai family (Orai 1, Orai 2, and Orai 3) can also contribute to the regulation of store-independent Ca^{2+} entry pathways.

The activity of all these Ca^{2+} selective channels is tightly controlled by a number of different protein partners. The membrane environment defines micro/nanodomains that also regulate the spatiotemporal pattern of intracellular Ca^{2+} signals in response to extracellular signals. However, under various pathological conditions Ca^{2+} entry can be observed under basal conditions. Basal Ca^{2+} influx has been observed in various cancer cells and it was suggested to contribute to deregulation of intracellular Ca^{2+} signaling supporting the cancer phenotype. [217–219]

3. Role of calcium signaling

Ca^{2+} signaling is used throughout the life history of an organism. Life begins with a surge of Ca^{2+} at fertilization and this versatile system is then used repeatedly to control many processes during development and in adult life. Ca^{2+} signaling plays a critical role in regulating a plethora of diverse processes such as proliferation, development, learning and memory, contraction and secretion. The versatility of Ca^{2+} signaling mechanisms emerges from the use of an extensive molecular repertoire of signaling components that can be assembled in combinations to create signals with widely different spatial and temporal profiles. Additional variations are achieved through the interactions that Ca^{2+} makes with other signaling pathways and thus regulates diverse cellular processes.

3.1. Cell proliferation

Cell proliferation is orchestrated through diverse proteins related to Ca^{2+} signaling inside the cell. [220–222] The role of calcium signaling in cell proliferation has been widely studied in T cells, where upon antigen binding to the T cell receptor, inositol-1,4,5-trisphosphate is generated which releases Ca^{2+} from the ER stores. Emptying of this store activates SOCs and subsequent sustained influx of extracellular Ca^{2+}. Ca^{2+} initiates the proliferative response by stimulating various transcription factors such as NF- kB, NF-AT and CREB. [211,223,224]

3.2. Transcription factor activation

The versatility of Ca^{2+} signaling lies in its ability to activate transcription factors either in the cytoplasm or within the nucleus. One function of Ca^{2+} signaling is to stimulate the Ca^{2+} sensitive protein phosphatase calcineurin to dephosphorylate NF-AT, which then enters the nucleus and mediates gene transcription. As soon as Ca^{2+} signaling stops, NF-AT is rapidly phosphorylated by nucleic kinesis, leaves the nucleus and the transcription of NF-AT-responsive genes ceases. [225] Another transcription factor regulated by the Ca^{2+} signaling is EGR-1. An increase of the intracellular Ca^{2+} concentration, by activating ionotropic or protein G-coupled receptors or voltage-gated L-type Ca^{2+} channels is often the prerequisite for the biosynthesis of EGR -1 and the transcription of EGR-1 target genes.

3.3. Calcium and apoptosis

Although calcium influx is involved in the regulation of many cellular functions, a sustained and excessive increase in the cytosolic Ca^{2+} levels leads to the activation of the intrinsic apoptotic pathway and cell death. Ca^{2+} induced cell death often depends on an interplay between the mitochondria and the ER. [226] Pro-apoptotic stimuli can influence how mitochondria respond to this periodic flux of Ca^{2+}. The Ca^{2+} signals produced by inositol-

1,4,5-trisphosphate are handled normally, but when superimposed on a background of pro-apoptotic stimuli they induce apoptosis through formation of the permeability transition pore. The latter usually forms when the mitochondria become overloaded with Ca^{2+} and so release cytochrome c. [227] Additionally, the apoptosis regulatory proteins that function either as death antagonists (Bcl-2 and Bcl-XL) or death agonists (Bax, Bak and Bad), may exert some of their actions by interfering with the Ca^{2+} dynamics between the mitochondria and the ER. Bax and Bad can accelerate opening of the permeability transition pore and so contribute to the release of cytochrome c. [228] Conversely, Bcl-2 which is located both in the ER and in mitochondria, seems to block Ca^{2+} induced apoptosis by enhancing Ca^{2+} storage [229,230]

3.4. Ca^{2+} dysregulation and cancer.

Malignant transformation is accompanied by changes in Ca^{2+} homeostasis that cause dysfunctions in the signs of proliferation and/or death. In order to proliferate at high rates, to increase cell motility and invasion, to escape death, to escape immune-attack, or to cause neovascularization, tumors remodel their Ca^{2+} signaling network. There is increasing evidence that tumorigenic pathways are associated with altered expression level or abnormal activation of Ca^{2+} channels. [218,231,232] For example, the expression of a novel glioma-specific T-type Ca2+-channel splice variant, Cav3.1ac, has been described in human glioma. This variant was detected in biopsy samples and some glioma cell lines, but not in normal brain or fetal astrocytes, suggesting that it might contribute to tumor pathogenesis. [233] As the expression pattern of Ca^{2+} channels and the degree of their functionality change in cancer, they may be useful for diagnostic purposes. A good example of this is the highly Ca^{2+} selective TRPV6, the expression and function of which was shown to correlate with prostate cancer grade. [234,235] Several studies demonstrate that the major calcium channels STIM1 and Orai1 have roles in proliferation and migration of various cancer cell lines. STIM1 knockdown is associated with inhibition of colon cancer cell migration and serum-induced migration in breast cancer, a disassembly and turnover of focal adhesion in hepatocellular

carcinoma and inhibition of proliferation in melanoma cells. [236–239] It has recently been demonstrated that SOCE promotes migration of colon cancer cell following the formation of a lipid raft ion channel complex composed of TRPC1/Orai1 channels and calcium-activated potassium channel SK3. [236] The formation of this TRPC1/Orai1/SK3 complex was simulated by EGR induced phosphorylation of STIM1 and activation of AKT pathway. Additionally, it has been shown that anti-EGFR mAbs can modulate SOCE and cancer cell migration, indicating that targeting the calcium signaling might modify the response to anti-EGFR mAbs.

4. Calcium channel blockers

Calcium channel blockers (CCB) are small molecules interacting directly with calcium channels and thus inducing calcium entry blockade. [240] Although the currently available calcium antagonists are chemically diverse, they share the common property of blocking the transmembrane flow of calcium ions through voltage-gated L-type and T-type channels. [240] Current forms of CCB include phenylalkylamines (verapamil, D600), dihydropyridines (nifedipine, nimodipine, nisoldipine, nicardipine) and benzothiazepines (diltiazem). These drugs are among the most commonly used agents for the treatment of cardiovascular diseases. [241] They have been proven effective in patients with hypertension, angina pectoris, and cardiac arrhythmias and may be beneficial in patients with left ventricular diastolic dysfunction, Raynaud's phenomenon, migraine, preterm labor, esophageal spasm, and bipolar disorders. [241]

Recent studies have demonstrated that CCB can impact cancer development and therapy. Several observational studies have associated the use of calcium channel blockers with an increased risk of breast cancer. The hypothesized mechanism for CCB increasing the risk of developing cancer is through decreased influx of calcium, thereby inhibiting apoptosis. [242,243] An association between the CCB nifedipine and an increased proliferation and migration of breast cancer cells has been reported in a few studies as well. [244,245] Additionally, treatment with verapamil has also been associated with

increased colon cancer mortality in epidemiologic studies. This could be explained by the fact that human colon cancer cells express L-type calcium channels that mediate calcium influx and apoptosis. [246] Other studies, on the contrary, have reported that CCB can be beneficial in the treatment of certain cancers associated with hypercalcemia and drug-resistant tumors. Nifedipine in combination therapy with cisplatin has been found to induce apoptosis in multidrug-resistant human glioblastoma cells. [247] Verapamil has been found to have a synergistic effect with 5-fluorouracil in metastatic colon adenocarcinoma cell lines. [248] Finally, due to the complexity of calcium signaling in normal and tumor cells, the effect of CCB in tumor development and treatment can be highly variable, depending on the experimental model and dose of drug used.

AIMS OF THE STUDY

Anti-CD20 mAbs are an essential component of the treatment of patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL). They mediate their antitumor effects by either activating the immune system or by direct apoptotic signaling in target cells.

In this study we focused on the direct apoptotic signaling induced by anti-CD20 antibodies. In a previous preclinical study, we have shown that in B cells exposed to anti-CD20 mAbs, rituximab and GA101, the expression of EGR-1 was strongly upregulated. Therefore, we hypothesized that EGR-1 could play an important role in the direct mechanism of action of anti-CD20 antibodies. We found that EGR-1 acts as a key player in the direct cytotoxic effect of anti-CD20 mAbs, and therefore could be a potential biomarker to predict the response to anti-CD20 therapy. Additionally, as EGR-1 is a calcium-regulated transcription factor, and CD20 is involved in the regulation of calcium flux, we investigated the role of the calcium influx in the antitumor activity of anti-CD20 mAbs. We found that the calcium influx induced by the anti-CD20 antibodies plays a crucial role for their antitumor effect, as calcium channel blockers impaired their efficacy.

These results could be of a major clinical importance, as it could lead to a better use of anti-CD20 antibodies and patient benefit. Additionally, pharmacological modulation of EGR-1 and the calcium flux may contribute to enhanced antitumor activity of anti-CD20 monoclonal antibodies.

RESULTS

Calcium channel blockers impair the antitumor activity of anti-CD20 monoclonal antibodies by blocking EGR-1 induction

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Abstract:

Anti-CD20 monoclonal antibodies (mAbs) are an essential component of the treatment of patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL). They mediate their antitumor effects by activating the immune system or by direct apoptotic signaling in target cells. In a previous preclinical study, we showed that treatment with anti-CD20 mAbs, rituximab and GA101, resulted in upregulated expression of early growth factor 1 (EGR-1) (*Dalle et al. 2011*). EGR-1 is a calcium (Ca^{2+}) regulated transcription factor and CD20 is hypothesized to regulate transmembrane Ca^{2+} flux. Therefore, we aimed to assess the role of EGR-1 and Ca^{2+} flux in the cytotoxic activity of anti-CD20 mAbs. We have shown that EGR-1 expression is rapidly upregulated in $CD20^{+}$ cells following rituximab and GA101 exposure. Decreasing EGR-1 expression by shRNA abolishes the direct cytotoxic effect of GA101 both *in vitro* and *in vivo*, indicating that EGR-1 is required for CD20-mediated cell death. Additionally, the overexpression of EGR-1 enhances the cytotoxic activity of GA101 both *in vitro* and *in vivo*. Furthermore, our results indicate that anti-CD20 mAbs induce calcium influx. Blocking the Ca^{2+} flux with calcium channel blockers (CCB) abolishes EGR-1 induction and impairs the GA101 efficacy *in vivo* and *ex vivo* in CLL blood samples. More importantly, our data indicate that patients receiving CCBs and anti-CD20 therapy have worst progression free survival and overall survival. In conclusion we have identified EGR-1 as a potential biomarker to predict response to anti-CD20 therapy. We demonstrated that co-treatment with CCBs negatively impacts the outcome of patients receiving anti-CD20 mAbs.

INTRODUCTION

Anti-CD20 monoclonal antibodies (mAbs) are an essential component of the treatment of patients with CD20-positive non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). The mechanisms of action of anti-CD20 mAbs involve complement activation and antibody dependent cellular toxicity, as well as direct apoptotic signaling in target cells. [1] Previously, we reported that the treatment of B-lymphoma cell lines with anti-CD20 mAbs, rituximab and GA101, resulted in upregulated expression of the transcription factor early growth factor -1 (EGR-1), suggesting its possible implication in the direct cytotoxic effect of these biomolecules. [2] EGR-1 is an immediate early gene encoding a transcription factor, member of the zinc-finger transcriptional factors family, which also includes three other members: EGR-2, -3 and -4. [3–5] With low basic level of expression, EGR-1 expression rapidly increases in response to a broad variety of molecular and environmental stimuli including growth factors, cytokines, shear stress, oxygen deprivation and an elevation of the intracellular Ca^{2+} concentration. [6–10] Many biological functions have been attributed to EGR-1, spanning from regulation of cell growth and proliferation to apoptosis induction. [6,11] However, the role of EGR-1 in response to passive immunotherapies or upon CD20 stimuli has not been explored so far.

Both, rituximab and GA101, target the B cell antigen CD20. CD20 is expressed on the surface of B cells, starting from pre-B stage to mature B-lymphocytes, but is lost upon differentiation into plasma cells. [12,13] No natural ligand has been identified for CD20, but due to its transmembrane structure, it is believed to be a component of a multimeric cell surface complex

that regulates Ca^{2+} transport across the plasma membrane. [14,15] Additionally, previous studies have shown that the rituximab ligation to CD20 results in increased intracellular calcium content, as it causes CD20 aggregation in the lipid rafts. [16] GA101, on the other hand, as a type II monoclonal antibody is believed to not induce calcium influx. The role of calcium flux in the antitumor activity of anti-CD20 antibodies still remains elusive.

We therefore set out to investigate the role of EGR-1 and Ca^{2+} flux in the direct cytotoxic activity of anti-CD20 mAbs, using NHL cell line, mice models and fresh patient samples. We show that the anti-CD20 antibodies mediate calcium influx and that the consequent increase in intracellular calcium content is accompanied by induction of EGR-1. Furthermore, EGR-1 acts as a key factor for the direct cytotoxic effect of anti-CD20 antibodies, as decreasing EGR-1 expression by shRNA abolished the direct cytotoxic effect of anti-CD20 antibodies. Finally, blocking Ca^{2+} flux with the calcium channel blockers inhibited EGR-1 induction by anti-CD20 mAbs, and thus impaired their anti-tumor activity.

RESULTS

EGR-1 is a downstream effector of CD20 signaling pathway

In a previous preclinical study by our team, transcriptome analysis have reveled EGR-1 expression strongly induced in B cells exposed to anti-CD20 antibodies, rituximab and GA101. [2] To investigate if EGR-1 is involved in CD20 signalization, we evaluated EGR-1 expression upon exposure to rituximab and obinutuzumab, in several different human NHL cell lines, as well as in breast cancer cell line MCF7 endogenously expressing CD20. We observed a strong EGR-1 induction at both mRNA and protein level, in human NHL cell lines as well as in MCF7 $CD20^{+}$ cells, while it remained unchanged in control MCF7 cells exposed to rituximab or GA101. (Figure 1A and 1B) EGR-1 induction by anti-CD20 mAbs could be detected as early as 1h, with a strongest expression at 3h and 6h. (Supplementary figure 1A) In parallel, we investigated the direct cytotoxic effect of GA101 and rituximab in the same cell lines. The 24h exposure to rituximab and GA101 induced cell death in NHL cell lines and MCF7 CD20+ cells. (Figure 1C) Anti-CD20 mAbs did not affect the viability of control MCF7 cells, confirming that the cell death induction is CD20 dependent. Moreover, GA101 showed significantly higher cytotoxic effect in comparison to rituximab. Comparable results were obtained using other NHL cell lines, RL, Raji and Daudi, exposed to anti-CD20 antibodies (Supplementary figure 1B and 1C)

These results indicate that anti-CD20 mAbs induce EGR-1 expression. This EGR-1 upregulation as well as the direct cytotoxic effect of anti-CD20 antibodies is dependent on the CD20 signalization.

EGR-1 is required for the direct cytotoxic effect of GA101 *in vitro* and *in vivo*

To examine whether EGR-1 is directly involved in the direct cytotoxic effect of anti-CD20 antibodies we have developed B-lymphoma cell models in which we either overexpressed EGR-1 or we knocked-down EGR-1 expression with a shARN. EGR-1 overexpression in Granta cell line resulted in an enhanced cytotoxic effect of GA101. (Figure 2A). On the other hand, the EGR-1 knocked down in Namalwa cells completely abolished the cytotoxic effect of GA101. (Figure 2B). To further confirm the importance of EGR-1 in GA101 cytotoxicity, we rescued EGR-1 expression in knocked-down cell lines. Rescuing EGR-1 expression restored cell's sensitivity to GA101 cytotoxic effect. (Figure B) Rituximab had a weak effect on cell viability, and it was not influenced by EGR-1 modulation. These results indicate that the expression of EGR-1 is required for the direct antitumor effect of GA101 *in vitro*.

Similar results supporting the importance of EGR-1 expression for the anti-tumor activity of anti-CD20 antibodies were obtained in xenografts in SCID mice. Mice with Granta xenografts overexpressing EGR-1 responded significantly better to GA101 therapy, in comparison to control group treated with GA101. (Figure 2C) The overexpression of EGR-1 did not affect the tumorigenesis nor the tumor growth and progression (data not shown). EGR-1 remained overexpressed in Granta EGR-1 tumors, and its expression was induced upon treatment with GA101. (Supplementary figure 2A) On the other hand, mice with Namalwa EGR-1 knocked down xenografts did not respond to GA101 therapy, while in control mice the treatment with GA101 significantly slowed down tumor progression. (Figure 2D) Interestingly, the tumorigenesis was faster when EGR-1 expression was knocked down, while

the tumor progression rate remained similar for untreated control and untreated shEGR-1 tumors (data not shown). EGR-1 remained low in Namalwa shEGR-1, and it was induced in mice treated with GA101. (Supplementary figure 2B) Additionally, mice with tumors in which we restored EGR-1 expression regained sensitivity to GA101 therapy, confirming that EGR-1 is essential for the antitumor activity of GA101. (Figure 2D)

Taken together, these date indicate that EGR-1 expression is also induced *in vivo* by anti-CD20 mAbs and support the fact that EGR-1 is a key factor for the direct cytotoxic effect of GA101, both *in vitro* and *in vivo*.

Anti-CD20 mAbs efficacy does not correlate with EGR-1 expression in primary chronic lymphocytic leukemic cells

To further investigate if anti-CD20 mAbs were able to upregulate EGR-1 expression in primary leukemic cells and if EGR-1 expression correlates with their efficacy, blood samples from CLL patients were exposed *ex-vivo* to anti-CD20 antibodies. Immune effector cells are present in blood samples, so in order to specifically investigate the direct cell death induction, we worked with immune effector inactive variants of GA101: GA101 P329G and GA101 P151F P329G. The variant GA101 P329G was generated by introducing a mutation in the human IgG1-Fc part of the antibody, resulting in completely abolished Fc γ R and C1q binding and retained cell death inducing properties. The second variant GA101 P151F P329G, has in addition to the P329G, a second mutation P151F resulting in enhanced cell death inducing properties. (Supplementary figure 3A) Both variants were able to induce EGR-1

expression *in vitro*: GA101 P329G at a similar level as GA101, and with enhanced expression by GA101 P151F P329G. (Supplementary figure 3B).

In CLL blood samples, the B-cell depletion by GA101 and GA101 P329G was at a similar rate, while GA101 P151F P329G presented more potent B-cell depletion *ex-vivo*. (Figure 3A) These results indicate that the direct cell death induction is the major mechanism of action of GA101 in blood samples ex-vivo. Interestingly, GA101 and its variants had a superior efficacy compared to rituximab, indicating that even in absence of immune effector mediated cytotoxicity, GA101 has a superior anti-tumor activity than rituximab. In parallel, we studied the baseline level of EGR-1 expression as well as upon exposure to anti-CD20 antibodies. We observed an important variation in EGR-1 baseline expression among CLL patients, as well as in the response to anti-CD20 mAbs. (Figure 3B and 3C). In the majority of the patients (53%), the expression of EGR-1 was induced by GA101, while in 19% of the patients it remained unchanged. Surprisingly, we did not find any correlation between EGR-1 baseline level of expression or EGR-1 induction and CLL cells depletion by anti-CD20 mAbs. (Supplementary figures 4).

Anti-CD20 mAbs induce calcium influx to upregulate EGR-1 expression

We next examined how anti-CD20 antibodies induce EGR-1 expression. Several studies indicate that CD20 is likely involved in the regulation of calcium influx, and rituximab binding could induce intracellular calcium influx. [15–17] It has previously been described that EGR-1 upregulation could be result of receptor operated Ca^{2+} channel stimulation and consequently an elevation of the intracellular Ca^{2+} concentration. [10] We determined whether

an induction of calcium influx is involved in the upregulated EGR-1 expression by anti-CD20 antibodies.

Calcium influx studies have shown an increased intracellular Ca^{2+} concentration in presence of GA101 in combination with a suboptimal dose of ionomycine, when compared to ionomycine alone. (Figure 4A). The increase in intracellular calcium resulted in enhanced EGR-1 expression as shown on figure 4B. On the other hand, blocking Ca^{2+} flux with the calcium channel blocker nifedipine or the Ca^{2+} chelating agent EGTA abolished EGR-1 induction by anti-CD20 mAbs. (Figure 4A and 4B) These data support the fact that the binging of anti-CD20 mAbs could induce calcium influx and underline the importance of calcium influx for the upregulation of EGR-1.

We next examined whether the increase in intracellular figure 4B concentration following the binding the binding of anti-CD20 antibodies was due to the induction of constitutive or capacitive calcium influx. Figure 4C shows that only GA101 promoted constitutive calcium influx. When we looked into the capacitive calcium influx, following a discharge of intracellular calcium stores, both rituximab and GA101 included calcium influx. (Figure 4D) Interestingly, rituximab exhibited higher capacitive calcium influx than GA101.

These data show that both rituximab and GA101 induce calcium influx, but through different mechanisms: rituximab contributes only to capacitive calcium influx, while GA101 contributes mainly to the constitutive, but is also able to induce constitutive calcium influx.

Nifedipine impairs GA101 antitumor activity *in vivo*

Our results have shown that calcium channel blocker nifedipine inhibits the calcium influx induced by GA101 and thus EGR-1 upregulation. As nifedipine is widely used in clinic for the treatment of cardiovascular diseases, we wondered if it could interfere with GA101 efficacy. SCID mice xenograffed with Granta tumors were pretreated with nifedipine two days before starting GA101 treatment, and continuously treated following the injection of GA101. Co-treatment with nifedipine impaired GA101 efficacy against the established Granta tumors, confirming the importance of the calcium influx for the direct antitumor activity of anti-CD20 antibodies. (Figure 5). The treatment with nifedipine alone showed slight but insignificant effect on tumor progression. This result indicates that the calcium influx is crucial for direct cytotoxic effect of GA101 *in vivo*, and the co-administration of calcium channel blockers impairs the antitumor activity of GA101.

Calcium channel blockers interfere with GA101 efficacy in CLL patient samples

To further explore the impact of calcium channel blockers on GA101 efficacy, we exposed fresh CLL patient samples to nifedipine and investigated B-cell depletion by GA101 as well as EGR-1 induction. The presence of nifedipine, significantly abolished GA101 efficacy to induce B cell depletion *ex vivo* in CLL blood samples. (Figure 5A) Moreover, the presence of nifedipine inhibited the EGR-1 induction by GA101 in primary leukemic cells, confirming that the calcium influx is crucial for EGR-1 induction and thus the cytotoxic effect of GA101. (Figure 5B) Interestingly, the presence of nifedipine did not impair the capacity of GA101 to induce ADCC/P or CDC. (data not shown).

Similar results were obtained with other calcium channel blockers often used in clinic in elderly patients with cardiovascular issues. Nicardipine, verapamil and dilitazem, all impaired the antitumor effect of GA101 *ex vivo*, confirming the negative impact of these drugs on the antitumor activity of GA101. (Figure 5C)

Patient co-treated with calcium channel blockers and anti-CD20 mAbs have worst PFS and OS

The vast majority of CLL patients are elderly subjects with an average age at diagnostics of 72 years. Many elderly patients suffer from hypertension, and for this they are treated with calcium channel blockers (CCB). Given the encouraging data we obtained *in vivo* and *in vitro*, we analyzed the data from GA101 clinical phase III study GOYA, to examine if the treatment with CCB had aby impact on the outcome of patients treated with anti-CD20 therapy. Patients being treated with calcium channel blocker in parallel with anti-CD20 antibodies, rituximab and GA101, had a significantly decreased progression free survival (PFS) (Figure 6A and 6B) as well as overall survival (OS) (Figure 6C and 6D). These results confirm our previous observations that calcium channel blockers impair the anti-tumor activity of anti-CD20 antibodies. Additionally, to confirm that it is the CCB that have negative impact on PFS and OS, rather than the pathology they are prescribed for, we checked if other drugs used to treat the same pathology as CCB had an effect on anti-CD20 therapy. Results indicate that the co-treatment with angiotensin-converting-enzyme (ACE) inhibitors also used for the treatment of hypertension did not have any effect on PSF or OS of patients treated with anti-CD20 mAbs.

(Supplementary figures 5) This indicates that difference in PSF an OS is due to the effect of CCB on B-cell rather than the pathology they are used for.

DISCUSSION

Anti-CD20 monoclonal antibodies are an important part of the therapeutic regimen for patients with B-cell malignancies. Although anti-CD20 mAbs have led to marked improvement in treatment response and overall survival, there remains a proportion of patients who do not respond to this therapy. Importantly, these patients may suffer adverse effects without benefit, and this has prompted the investigation biomarkers to better define patient selection.

Recently, we have identified EGR-1 as one of the highest expressed genes in B cells exposed to anti-CD20 antibodies rituximab and GA101. [18] EGR-1 is a transcription factor with major functions in controlling cell proliferation, tumor suppression and apoptosis. [6,11] In the present study, we investigated the role of EGR-1 in the direct cytotoxic effect of anti-CD20 monoclonal antibodies. We confirmed that EGR-1 expression is rapidly upregulated in CD20+ cells following rituximab and obinutuzumab exposure, indicating that EGR-1 is a downstream target of CD20 signaling. Furthermore, EGR-1 upregulation enhanced by rituximab and GA101 leads to increased cell death. Indeed, we showed that EGR-1 is required for the CD20-mediated cell death both *in vitro* and *in vivo*, as decreasing EGR-1 expression by shRNA abolished the direct cytotoxic effect of GA101. The importance of EGR-1 in cell death induction by anti-CD20 mAbs was confirmed by EGR-1 rescue in EGR-1 knocked-down cells which restored sensitivity to GA101. Likewise, the overexpression of EGR-1 resulted in enhanced cytotoxic activity both *in vitro* and *in vivo*. However, although we were able to confirm that the expression of EGR-1 is induced in primary leukemic cells, surprisingly we did find any correlation between EGR-1 level of expression and the efficacy of anti-CD20

mAbs in CLL blood samples. This might be due to the fact that most of the blood samples came from patients who have already received treatment for their pathology. Therefore, a further study would be necessary to investigate the expression and induction of EGR-1 at diagnostic and a possible correlation with patient's outcome. In addition, we observed a strong variation in EGR-1 baseline expression among patients, which also did correlate with the efficacy of anti-CD20 mAbs. A recent study by Krysiak at al. showed that EGR-1 is recurrently mutated in patients with follicular lymphoma. [19] Hence, it would be interesting to investigate if this is also the case in chronic lymphocytic leukemia, and if the mutational status of EGR-1 would interfere with the response to anti-CD20 mAbs.

Several studies point out that CD20 point to the biological function of CD20 as regulator of calcium signals. Additionally, the binding of rituximab to CD20 induces increase in intracellular calcium content and EGR-1 is described as calcium regulated transcription factor. [10,16] In order to identify the mechanism through which the anti-CD20 antibodies induce EGR-1 expression, we investigated if the calcium could play a role in this mechanism. We demonstrate for the first time that GA101, as a type II antibody is also able to induce calcium influx. In fact, GA101 predominantly induces constitutive calcium influx while rituximab functions through capacitive calcium influx. More importantly, we demonstrated that the calcium entry and the consequent increased intracellular calcium levels are required for EGR-1 induction downstream apoptotic signaling. The importance of the calcium influx is highlighted by the presence of calcium channel blockers, which completely impaired the anti-tumor activity of ant-CD20 antibodies. Calcium channel blockers are drugs disrupt the movement of calcium through calcium

channels, and are often used as medications for cardiovascular diseases. In the phase III clinical study GOYA, more than 20% of the patients treated with anti-CD20 mAbs received CCB. We show that these patients have worst PFS and OS, indicating that CCB interfere with anti-CD20 mAbs. Our results highlight the importance of coordinating drugs administration in order to benefit the therapeutic potential of anti-CD20 antibodies. Although we did not find that CCB interfere with the other mechanism of action of anti-CD20 mAbs, several studies indicate that CCBs reduce NK cell mediated target cell lysis by reducing calcium-dependent degranulation, and thus potently impair the ADCC. [20] Additionally, CCB are shown to modulate immune reactions by suppressing the activation of T-cells, macrophages and reducing the cytokines production. [21]

By using *in vitro*, *in vivo* and *ex-vivo* approaches, we demonstrate, that anti-CD20 mAbs induce calcium influx, and the increase in calcium content induces EGR-1 expression, which plays a key role in cell death mediation in response to ant-CD20 mAbs. Finally, we showed that CCB by blocking the calcium flux, impair the antitumor activity of anti-CD20 antibodies, and decrease the PFS and OS of patients receiving anti-CD20 mAbs. Our study reveals the importance of coordinating drug administration, and reveals important adverse effects of CCB on anti-CD20 therapy.

MATERIAL AND METHODS

Cell lines and culture

Human non-Hodgkin's lymphoma cell lines Granta (mantle cell lymphoma), Namalwa (Burkitt's lymphoma) and breast cancer cell line MCF7 were all obtained from ATCC. NHL cell lines were grown in RPMI 1640 media and MCF7 cells were maintained in DMEM. Both media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured at 37˚C, in humidified atmosphere of 5% CO2.

Antibodies and reagents

The anti-CD20 antibody rituximab (Mabthera®, Roche) was obtained commercially. Anti-CD20 antibody GA101 (Gazyva/Gazyvaro®) and GA101 variants P239G and P151F P239G were kindly provided by Roche Glycart AG.

Calcium channel inhibitors nifedipine, nicardipine, verapamil and diltiazem were all obtained commercially form Sigma-Aldrich. The drugs were dissolved as per manufacturer's instructions and used at the concentrations indicated.

RT-PCR and quantitative real time PCR

The total RNA was extracted with RNeasy mini kit (Qiagene) according to the manufacturer's instructions. Extracted RNA was reverse transcribed into the cDNA using M-MLV Reverse Transcriptase kit (Invitrogen). The resulting cDNA samples were diluted 50-fold and used for quantitative real time PCR.

The following primers were used: EGR- 1 forward, 5′-GCCTGCGACATCTGTGGA-3′, EGR-1 reverse 5′ GCCGCAAGTGGATCTTGG-3′; ribosomal 28s forward 5′-GAAAGATGGTGAACTATGCC-3′, ribosomal 28S reverse 5′-TTACCAAAAGTAGCCCACTA- 3′. Real-time qPCR was performed on LightCycler Nano (Roche), detecting SYBR Green I signal transmission. The expression of genes was normalized to housekeeping gene ribosomal 28S. Relative expression was determined using the ΔCt method.

Western blotting

Total protein extracts were prepared from subconfluent cultures by resuspending cells in RIPA-Buffer (100 mM Tris-HCl, pH 7.5, 750 mM NaCl, 5% Triton X100, 0.5% SDS, 5% sodium deoxycholate, protease inhibitor Cocktail (Roche), 0.1 mM DTT, 0.2 mM NaF) and incubated for 1h on ice. Following the incubation, cell lysates were centrifuged at 12,000 x g for 15 minutes at 4˚C and the supernatants were collected and assayed for protein concentration using Coomassie Plus protein assay kit (Thermo Scientific, PI-23236). Equal amounts of protein were loaded and separated on 10-15% SDS-PAGE acrylamide gels under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane using an electroblotting apparatus (Bio-Rad2). Membranes were incubated with the appropriate dilution of primary antibody, followed by incubation with IRDye secondary antibody. The following antibodies were used: rabbit polyclonal anti-EGR-1 (clone 588) 1:200 (Santa Cruz Biotechnologies), rabbit polyclonal anti-SUMO-1 1:1000 (Cell Signaling Technology), monoclonal anti-β actin (clone AC-15)

1:5000 (Sigma), goat anti-rabbit IRDye (LI-COR, Odyssey) and goat anti–mouse IRDye (LI-COR, Odyssey). The proteins were detected by chemiluminescence using the Odyssey infrared system (LI-COR Biotechnology). Analysis and quantification were performed with Odyssey™ software.

Cell transfections

Plasmids pCDNA3 CD20 and pCDNA3 EGR1 (Origene) and pSuperioir.puro shEGR1 were expanded from Escherichia coli DH5 and purified using the The PureLink® HiPure Plasmid Filter Maxiprep Kit (Invitrogen). MCF7 cells were transfected with pCDNA3 and pCDNA3 CD20 using lipofectin. Briefly, cells (200,000 cells per flask) were incubated for 5 h with 5 g of plasmid in presence of 12.5 g of lipofectin (Invitrogen). After 72 h of growth in complete medium stably transfected cells were selected with 0.8 mg/ml geneticine (Invitrogene). Granta cells were transfected with pCDNA3 and pCDNA3 EGR-1, and Namalwa cells were transfected with pSuperioir.puro shCTRL and pSuperioir.puro shEGR1 using ultrasounds as described previously. [22] Briefly, $5x10^6$ were resuspended in 650µl Opti-MEM® and 10µg plasmid was added to the suspension. After ultrasound application, cell suspensions were placed in complete medium cultured at 37°C in humidified atmosphere with 5% CO2 incubator for 72 h. Transfected Granta cells were selected with 0.7 mg/ml geneticine (Invitrogene). Transfected Namalwa cells were selected with 0.6 µg/ml puromycin (Invitrogene).

Cell death assays

Cell death induction was evaluated by annexinV and propidium staining evaluated using flow cytometry. Briefly, 1×10^6 cells per condition were incubated in presence or not of 10 μg/mL rituximab or GA101 for 24 hours at 37°C. Cells were stained using Annexin-V-FLUOS Staining Kit (Roche), according to the manufacturer's protocol. Dead and viable cells were discriminated by Annexin V/propidium iodide (PI) staining using flow cytometry LSRII (BD). Analysis was performed with BD FACSDiva™ software.

Calcium influx studies

Granta cells $20x10^6$/ml were incubated with 1.5mM calcium-sensitive dye indo 1-AM (Molecular Probes, Inc.) at 37°C for 45min. Cells were washed twice and resuspended in RPMI media. Following the assessment of basedline calium ratio, ionomycine (Invitrogen) 0,5-2,5 µM was added, and or 10µh/ml GA101. Cells were analyzed for calcium mobilization in BD-LSR (BD Biosciences). Values were plotted as ratio of fluorescence at Ca^{2+} free indo-1 and Ca^{2+} bound indo-1.

Intracellular Ca^{2+} measurements

Sticking cells were loaded in petri-dish for 45 min at 37 °C with the ratiometric dye Fura2-AM (5 μM). Then, cells were trypsinized, washed with Opti-MEM®Reduced Serum Mediumand centrifugated (x800g for 5min). Cells were suspended in PSS Ca^{2+} free solution and treated with thapsigargin (4 μM)

(T7458, Life-Technologies): to deplete the intracellular store depletion before injection of 2 mM of CaCl2. Fluorescence emission was measured at 510 nm using the monochromator of a spectrofluorimeter (Hitachi FL-2500, Ltd) with an excitation light at 340 and 380 nm. Maximum of fluorescence (Peak of calcium influx (F340/ F380)) after injection of 2 mM of Ca^{2+} is measured and normalized to control condition.

Animal Studies

All animal procedures were performed in accordance with the European Union Directive (86/609/EEC). Experiments were performed under individual permit and in animal care facilities accredited by the French Ministry of Agriculture. The study was approved by the local animal ethical committee.

Studies were conducted using female SCID CB17 mice (4 weeks old, 5 mice/group, Charles River). Mice were injected subcutaneously with $5x10^6$ NHL cells as indicated. GA101 was injected intraperitoneally at a dose of 30mg/kg once the tumor volume reached 100mm3. Nifedipine was injected intraperitoneally at a dose of 10mg/kg, 5 days a week. Tumor growth was monitored by caliper measurement of the tumor volume 3-5 times per week.

Fresh patient samples

Peripheral blood on EDTA was obtained from patients (aged 47- 85 years) diagnosed with CLL according to standard criteria. Procedures were

conducted under the approval of Lyon Hospital Ethics committee with all patients signing informed consent.

CLL cells were isolated by ficoll density-gradient centrifugation and purity was verified by CD20 labelling (≥ 70% CD20-positive). Whole blood samples or CLL cells (for PCR analysis) were exposed to anti-CD20 antibodies, in combination with or without calcium channel blocker for 24h. The B-cell depletion was evaluated following CD5 (Miltenyi), CD19 (BD Bioscience) and CD45 (BD Bioscience) staining using flow cytometry LSRII (BD). Analysis was performed with BD FACSDiva™ software.

Statistics

Statistical analyses have been performed using Prism software. Results were expressed as the mean ± SD of at least three independent experiments, each performed in triplicates.

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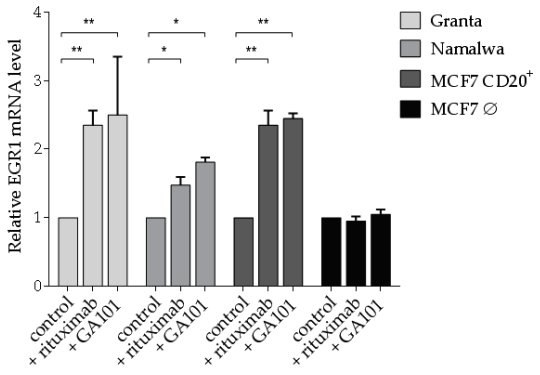
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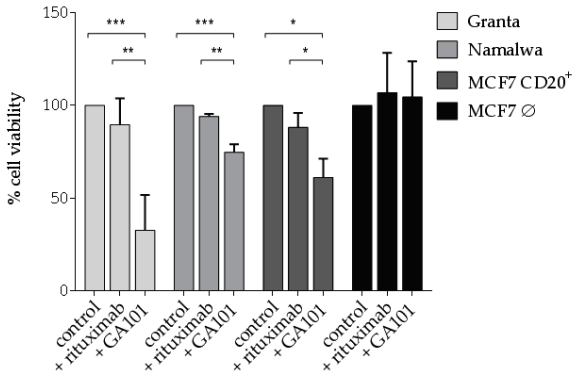
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Figure 1.

A.



C.



B.

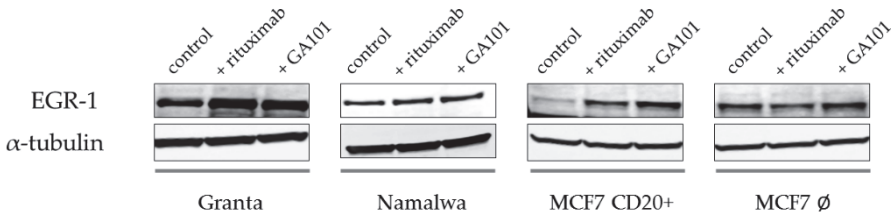


Figure 1. Anti-CD20 mAbs up-regulate EGR-1 expression and induce cell death only in CD20$^+$ cells. Analysis of EGR-1 mRNA expression by qRT-PCR (A) and protein expression by Western blot (B), in human NHL cell lines Granta and Namalwa, breast cancer cell line MCF7 cells stably transfected for CD20 expression (MCF7 CD20+) and MCF7 cells transfected with empty vector (MCF7Ø). The expression of EGR-1 was analyzed following 3h exposure to 10 µg/ml anti-CD20 mAbs. EGR-1 mRNA level is normalized to the expression of the housekeeping gene 28S. (C) Cell viability evaluated by annexinV/PI staining on flow cytometry following 24h exposure to 10 µg/ml anti-CD20 mAbs. The cell viability was normalized with control untreated cells at 100%. Values shown are means +- SD of the least 3 independent experiments. * $p < 0,05$, ** $p < 0,01$ *** $p < 0,005$

Figure 2.

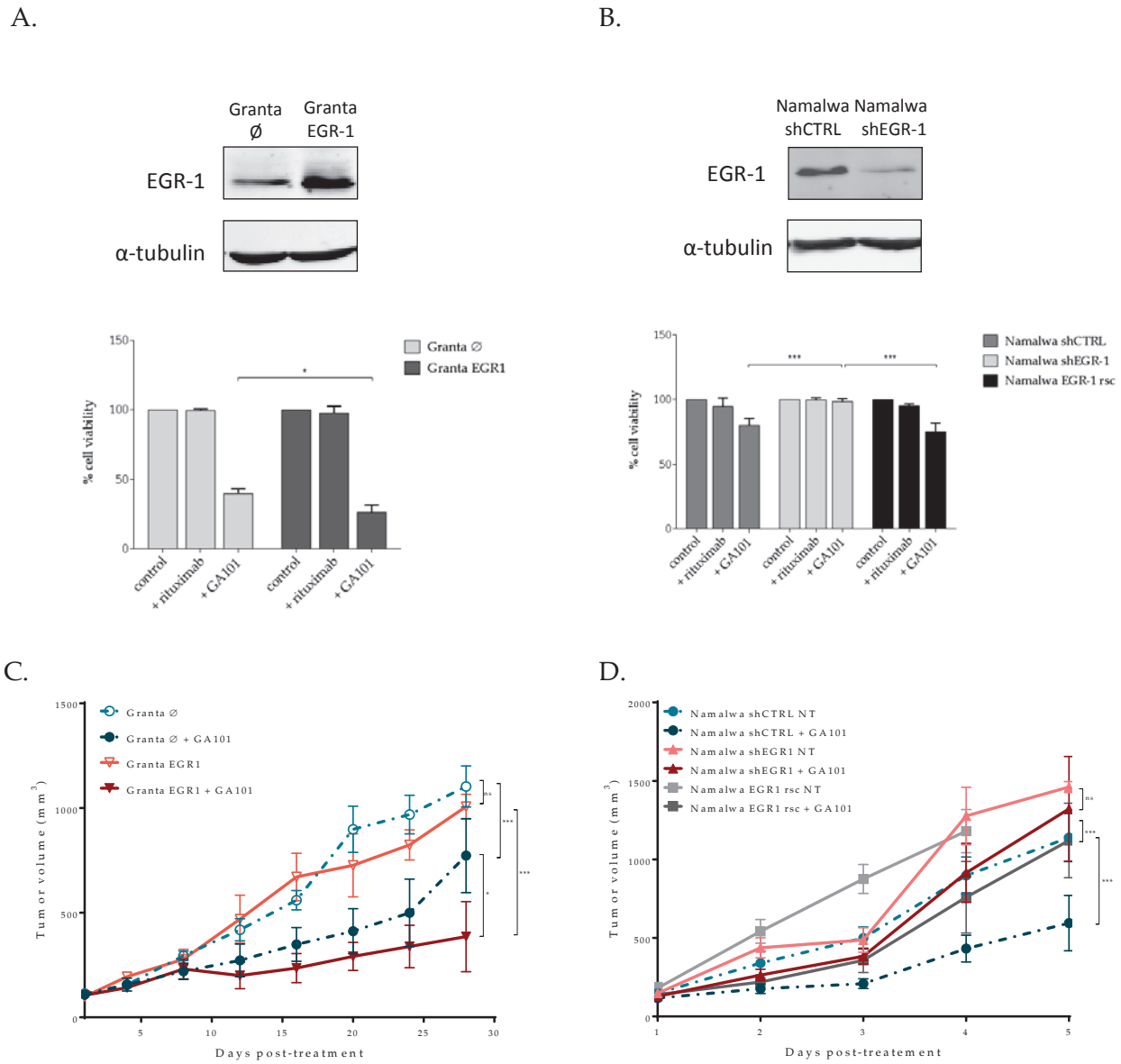


Figure 2. EGR-1 mediates GA101 cytotoxicity *in vitro* and i*n vivo*. **(A)** Mantle cell lymphoma cell line Granta was stably transfected with a pcDNA3 plasmid encoding for EGR1 (Granta EGR1) or empty vector (Granta Ø). **(A)** Burkitt's lymphoma cell line Namalwa was stably transfected with pSup CTRL (Namalwa shCTRL) or pSup EGR1 (Namalwa shEGR1) encoding for a shRNA targeting EGR1. Namalwa shEGR1 were further transfected with pcDNA3 plasmid encoding for EGR1 to restore EGR-1 expression (NamalwaEGR-1 rsc). Cell viability evaluated by annexinV/PI staining on flow cytometry following 24h exposure to 10 µg/ml anti-CD20 mAbs. The cell viability was normalized with control untreated cells at 100%. Values shown are means +- SD of the least 3 independent experiments. **(C)** Effects of the anti-CD20 mAb GA101 (30mg/kg/week) treatment on tumor size in SCID mice subcutaneously injected with cells overexpressing EGR -1 or **(D)** with cells knocked down for EGR-1 or EGR1 rescue (n = 5 mice for group. * $p < 0,05$, ** $p < 0,01$ *** $p < 0,005$

Figure 3.

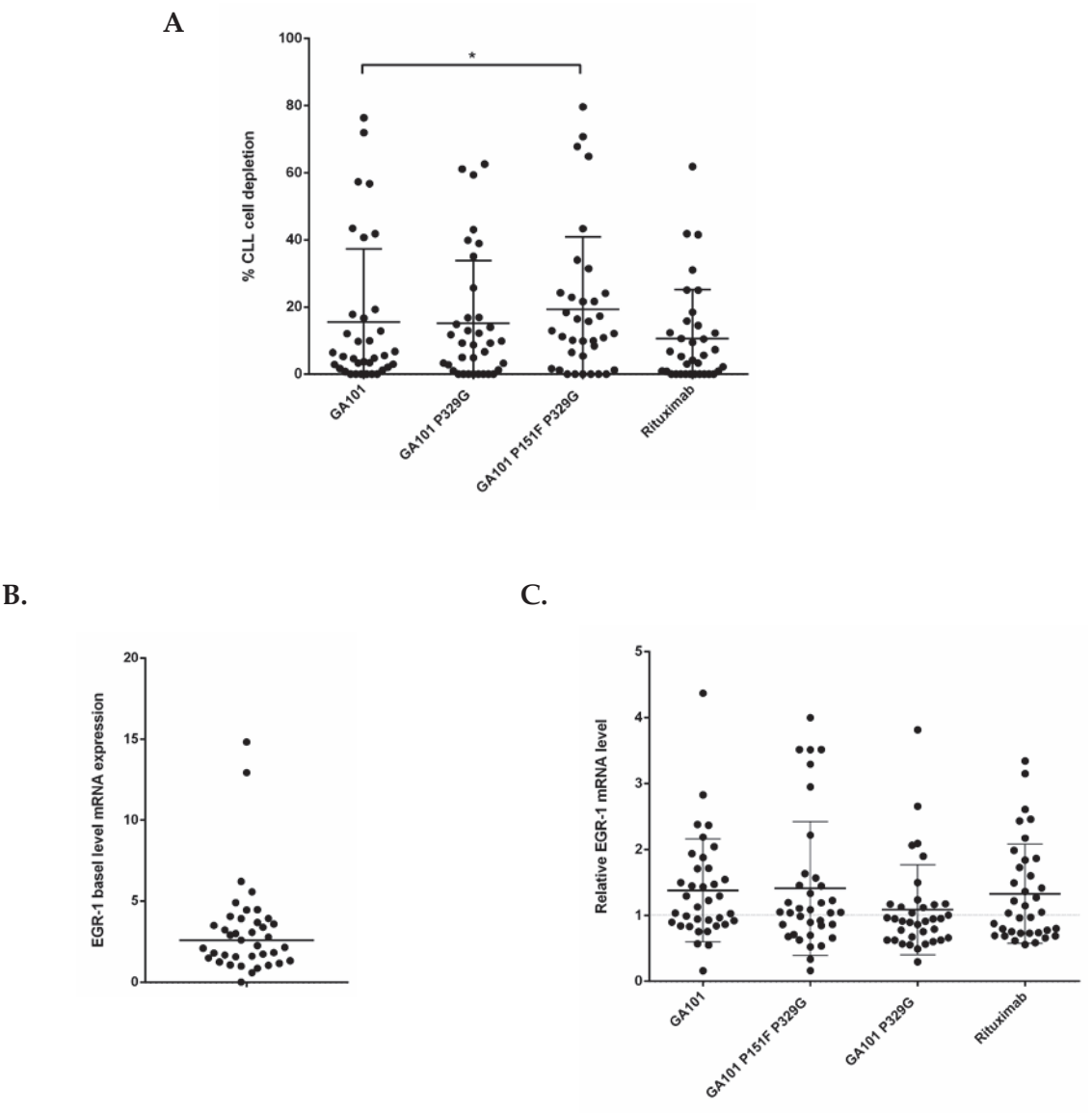
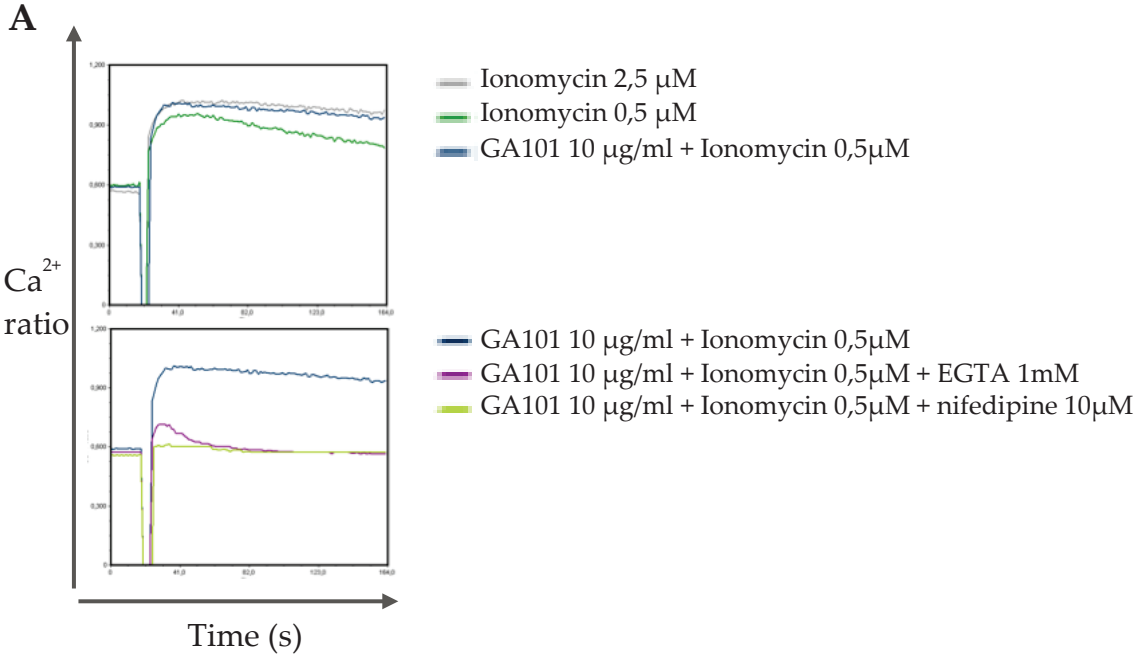


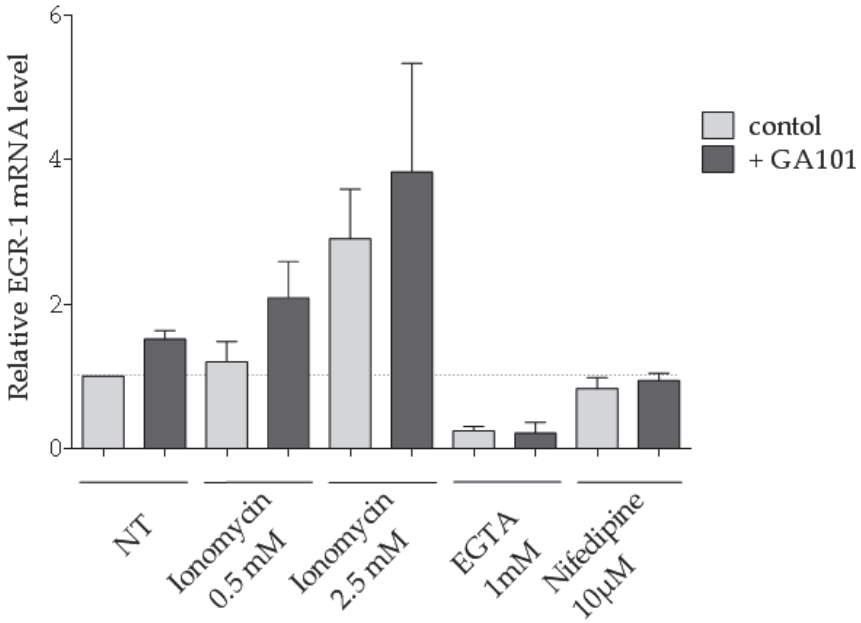
Figure 3. Anti-CD20 mAbs induce EGR-1 expression in primary leukemic cells (A) Blood samples form CLL patients were exposed to 10 µg/ml anti-CD20 mAbs for 24h. CLL-cell depletion was assessed following CD5, CD19 and CD45 staining on flow cytometry. The percent of depletion was normalized with untreated control sample to 100%. (B) PMBC from CLL blood samples were collected to assess EGR-1 base line expression by RT-qPCR or (C) were exposed to 10 µg/ml anti-CD20 mAbs for 3h to investigate EGR-1. EGR-1 mRNA level is normalized to the expression of the housekeeping gene 28S. n=35; * $p < 0,05$

Figure 4.

A


Ca2+ ratio
Time (s)
Ionomycin 2,5 μM
Ionomycin 0,5 μM
GA101 10 μg/ml + Ionomycin 0,5μM
GA101 10 μg/ml + Ionomycin 0,5μM
GA101 10 μg/ml + Ionomycin 0,5μM + EGTA 1mM
GA101 10 μg/ml + Ionomycin 0,5μM + nifedipine 10μM

B.


Relative EGR-1 mRNA level
6
4
2
0
contol
+ GA101
NT
Ionomycin 0.5 mM
Ionomycin 2.5 mM
EGTA 1mM
Nifedipine 10μM

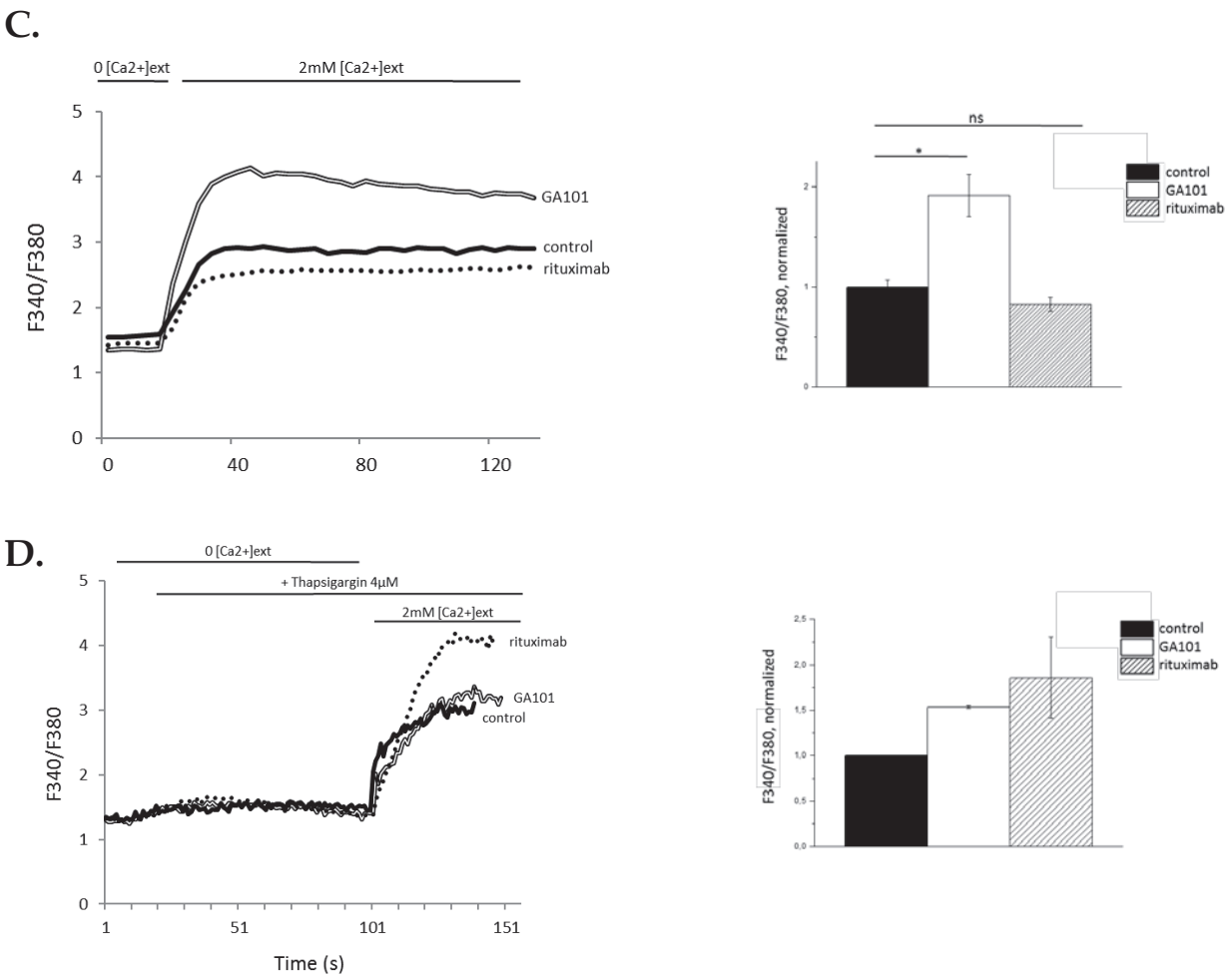


Figure 4. Calcium influx induced by anti-CD20 mAbs is necessary for EGR-1 induction. (A) Upper panel: Granta cells loaded with the fluorescent Ca2+ indicator dye Indo-1 AM were stimulated with GA101 and ionomycin at suboptimal dose (0,5mM) to potentiate the calcium flux or with high dose ionomycin (2,5mM) to raise the calcium influx. Lower panel: Granta cells loaded with the fluorescent Ca2+ indicator dye Indo-1 AM were stimulated with GA101 and ionomycin at suboptimal dose (0,5mM) and calcium channel blocker nifedipine (10µM) orcalcium chelating agent EGTA (1mM) to inhibit calcium influx. **(B)** Granta cells were exposed to ionomycine(0,5-2,5mM), nifedipine (10µM) or EGTA (1mM) in presence of not of 10 µg/ml GA101 for 3h. EGR-1 mRNA expression was evaluated by RT-qPCR and normalized to is normalized to the expression of the housekeeping gene 28S. **(C)** Fluorescence measurement (left panel) and histograms showing relative fluorescence (right panel) of Ca^{2+}entry in Granta cells following 1h incubation with anti-CD20 mAbs. **(D)** Fluorescence measurement (left panel) and histograms showing relative fluorescence (right panel) of Ca^{2+}entry following 1h incubation with anti-CD20 mAbs and after intracellular calcium store depletion by 4µM thapsigargin in Granta cells. Data are means ± SEM. *p<0.05

Figure 5.

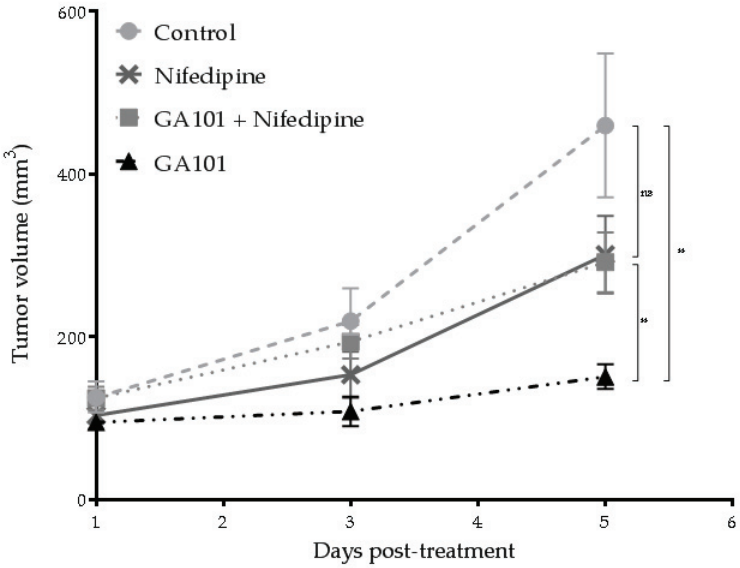
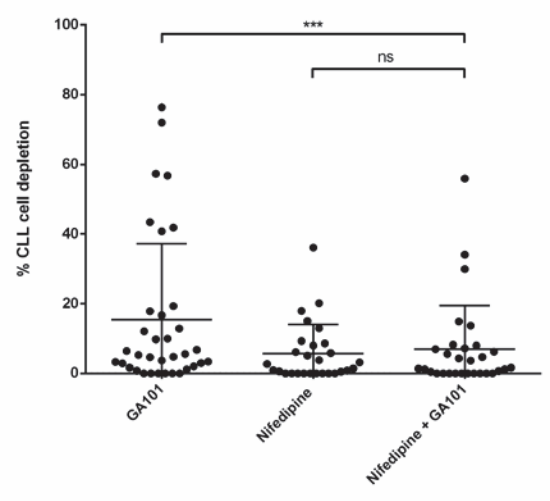


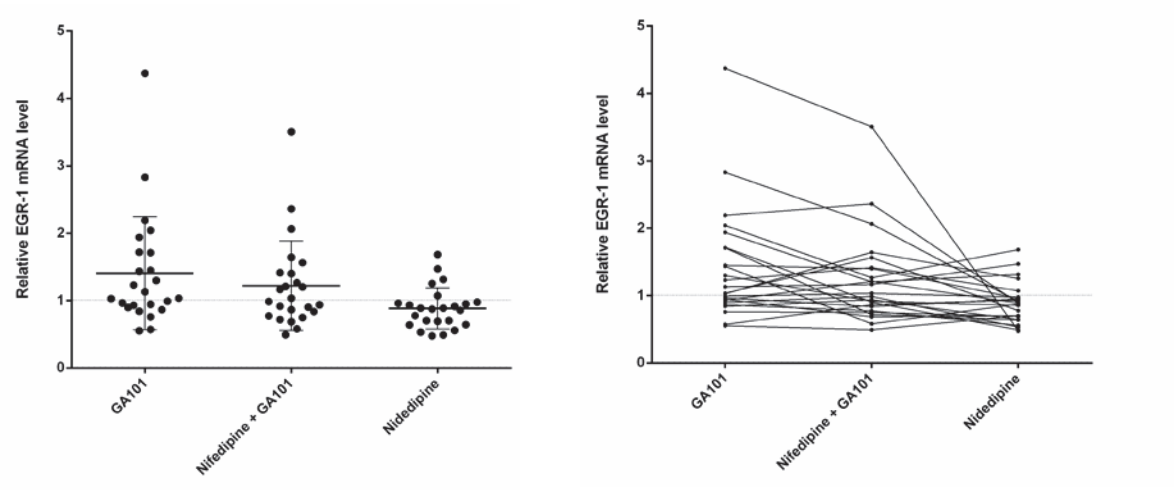
Figure 5. Calcium channel blocker Nifedipine impairs GA101 antitumor activity *in vivo*. SCID mice were subcutaneously injected with a human mantle cell lymphoma line Granta. Mice were treated GA101 (30mg/kg/week) and/or nifedipine (1mg/kg/day) once the tumor size reached 100mm3 (n = 5 mice for group).

Figure 6

A.


***
ns
% CLL cell depletion
100
80
60
40
20
0
GA101
Nifedipine
Nifedipine + GA101

B.


Relative EGR-1 mRNA level
5
4
3
2
1
0
GA101
Nifedipine + GA101
Nidedipine
Relative EGR-1 mRNA level
5
4
3
2
1
0
GA101
Nifedipine + GA101
Nidedipine

C.

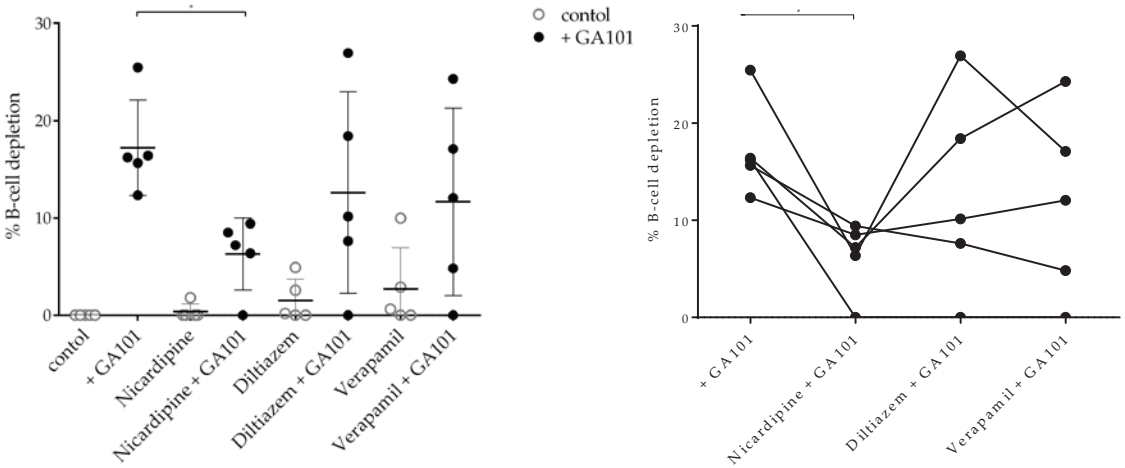
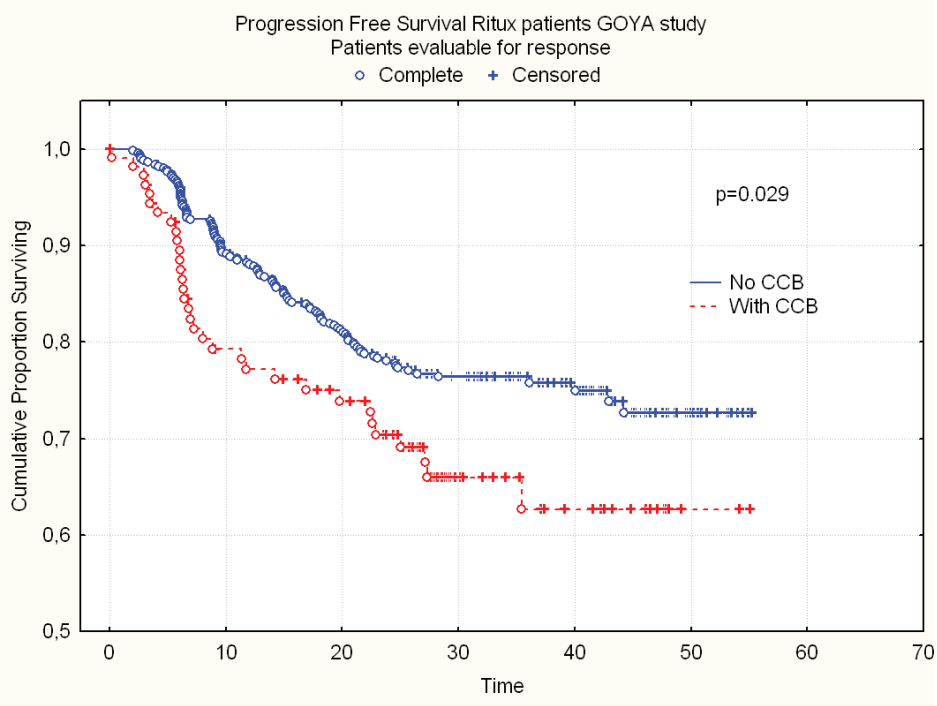

% B-cell depletion
30
20
10
0
contol
+ GA101
Nicardipine
Nicardipine + GA101
Diltiazem
Diltiazem + GA101
Verapamil
Verapamil + GA101
contol
+ GA101
% B-cell depletion
30
20
10
0
+ GA101
Nicardipine + GA101
Diltiazem + GA101
Verapamil + GA101

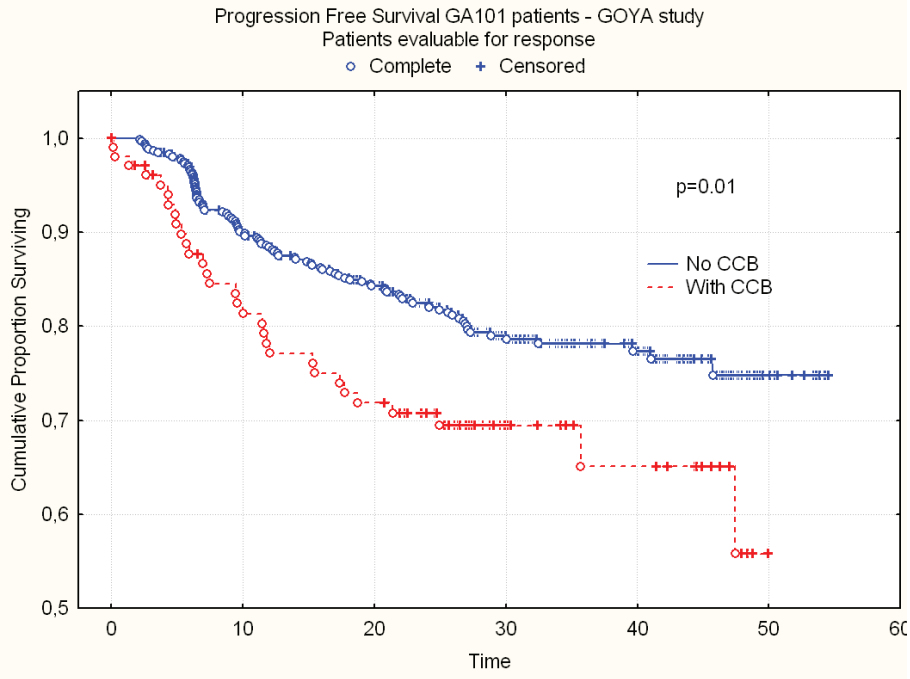
Figure 6. Calcium channel blockers reduce the antitumor activity of GA101 in primary leukemic cells. **(A)** Blood samples form CLL patients were exposed to 10 µg/ml GA101 and/or 10 µM nifedipine for 24h. CLL-cell depletion was assessed following CD5, CD19 and CD45 staining on flow cytometry. The percent of depletion was normalized with untreated control sample to 100%. **(B)** PMBC from CLL blood samples were exposed to 10 µg/ml GA101 and/or 10 µM nifedipine for 3h to investigate EGR-1 expression. EGR-1 mRNA level is normalized to the expression of the housekeeping gene 28S. n=24; **(C)** Blood samples form CLL patients were exposed to 10 µg/ml GA101 and/or 10 µM CCB nicardupine, diltiazem and verapamil. CLL-cell depletion was assessed following CD5, CD19 and CD45 staining on flow cytometry. The percent of depletion was normalized with untreated control sample to 100%. n=5 * $p < 0,05$; *** $p < 0,001$

Figure 7.

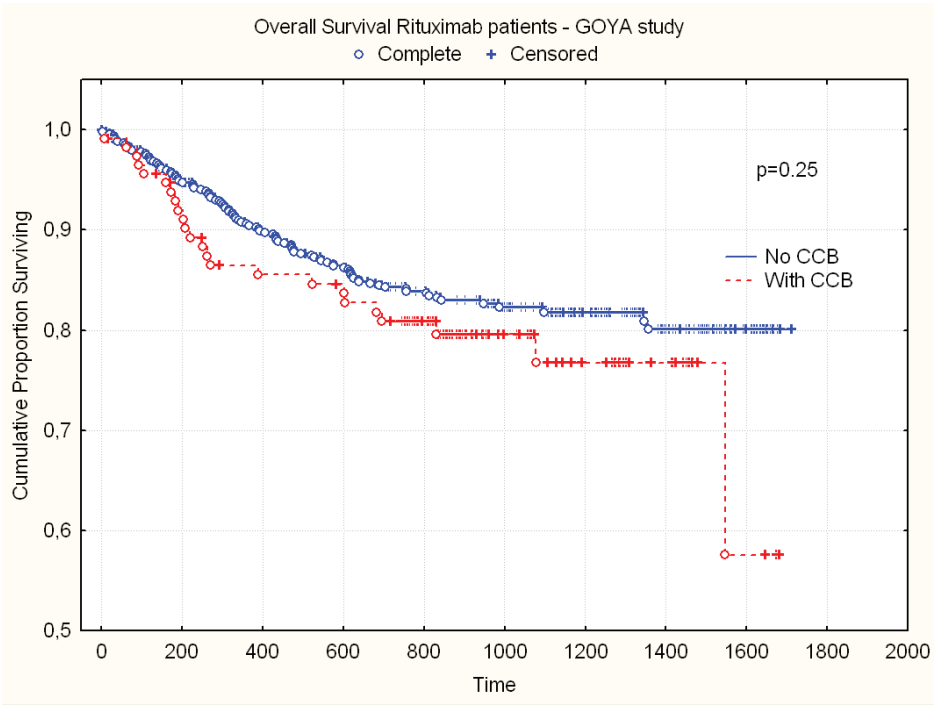
A.


Progression Free Survival Ritux patients GOYA study
Patients evaluable for response
Complete
Censored
Cumulative Proportion Surviving
1,0
0,9
0,8
0,7
0,6
0,5
p=0.029
No CCB
With CCB
0
10
20
30
40
50
60
70
Time

B.


Progression Free Survival GA101 patients - GOYA study
Patients evaluable for response
Complete
Censored
Cumulative Proportion Surviving
1,0
0,9
0,8
0,7
0,6
0,5
p=0.01
No CCB
With CCB
0
10
20
30
40
50
60
Time

C.


Overall Survival Rituximab patients - GOYA study
Complete
Censored
p=0.25
No CCB
With CCB
Cumulative Proportion Surviving
1,0
0,9
0,8
0,7
0,6
0,5
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Time

D.

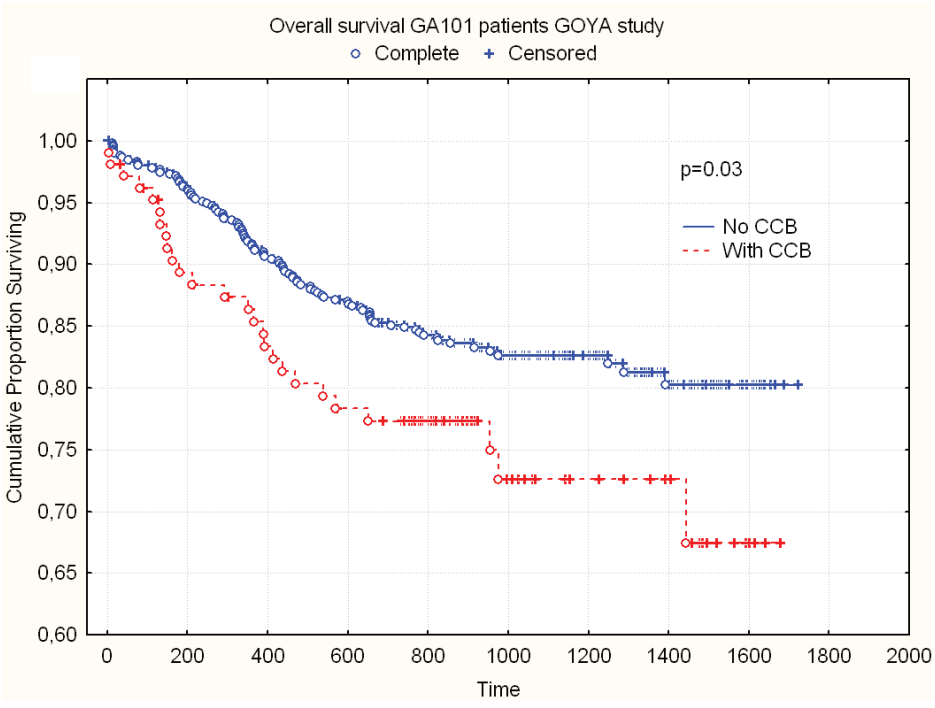
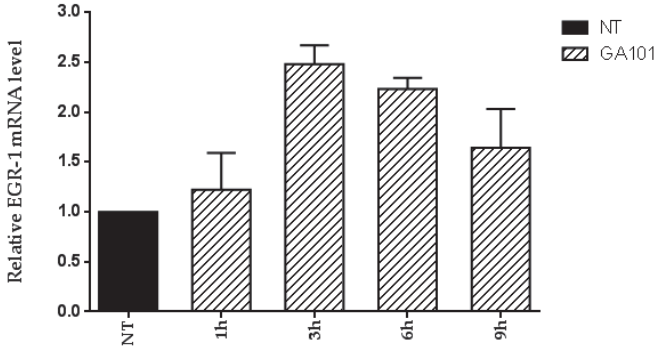

Overall survival GA101 patients GOYA study
Complete
Censored
p=0.03
No CCB
With CCB
Cumulative Proportion Surviving
1,00
0,95
0,90
0,85
0,80
0,75
0,70
0,65
0,60
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Time

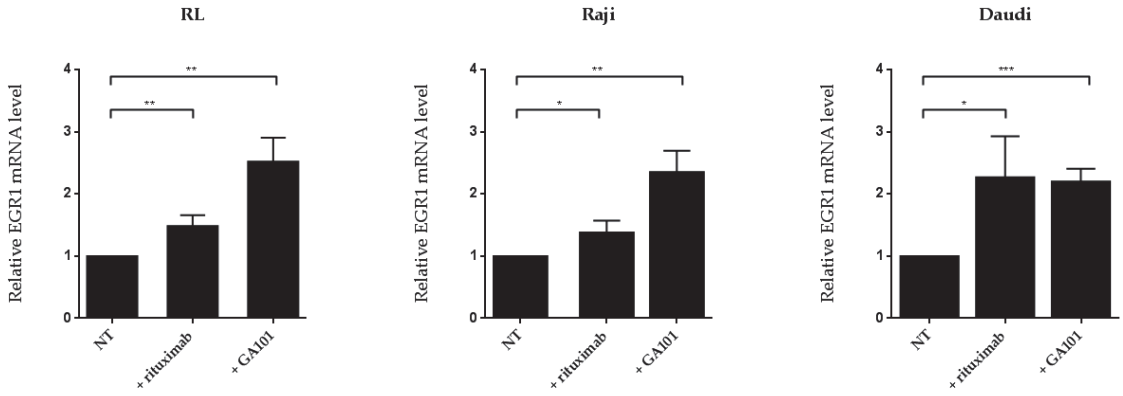
Figure 7. Calcium channel blockers decrease the PFS and OS of patients treated with anti-CD20 mAbs. Results from GOYA, an open-label, randomized, parallel group study evaluating the efficacy and safety of GA101 in combination with cyclophosphamide, doxorubicin, vincristine, and prednisolone or prednisone (CHOP) chemotherapy versus rituximab (MabThera/Rituxan) with CHOP in previously untreated participants with cluster of differentiation 20 (CD20)-positive diffuse large B-cell lymphoma (DLBCL). **(A)** Progression free survival curves of patients treated with rituximab or **(B)** GA101 taking or not calcium channel blocker. **(C)** Overall survival curves of patients treated with rituximab or **(D)** GA101 taking or not calcium channel blocker.

Supplementary Figure 1.

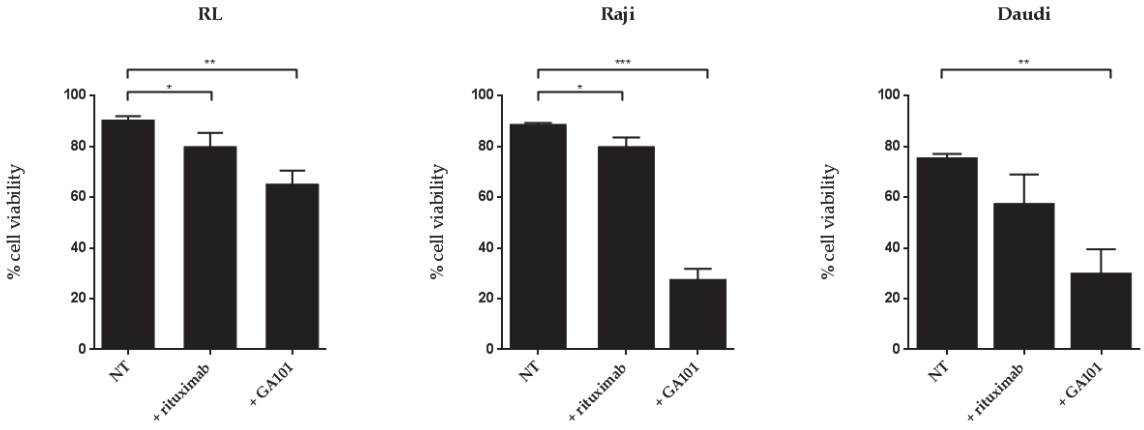
A.



B.

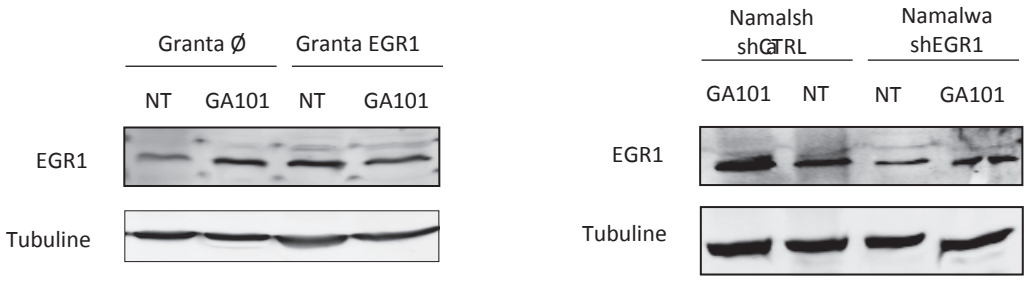


C.



Supplementary figure 1. Anti-CD20 mAbs up-regulate EGR-1 expression and induce direct cell death in NHL cell lines (A) Kinetics of EGR-1 mRNA expression, assessed by RT-qPCR shows highest EGR-1 expression at 3h exposure to 10µg/ml GA101 in Granta cells. **(B)** EGR-1 expression following 3h exposure and **(C)** cell viability were evaluated in NHL cell lines RL, Raji and Daudi, following 3h and 24h exposure to anti-CD20 antibodies, respectively. * $p < 0,05$, ** $p < 0,01$ *** $p < 0,005$

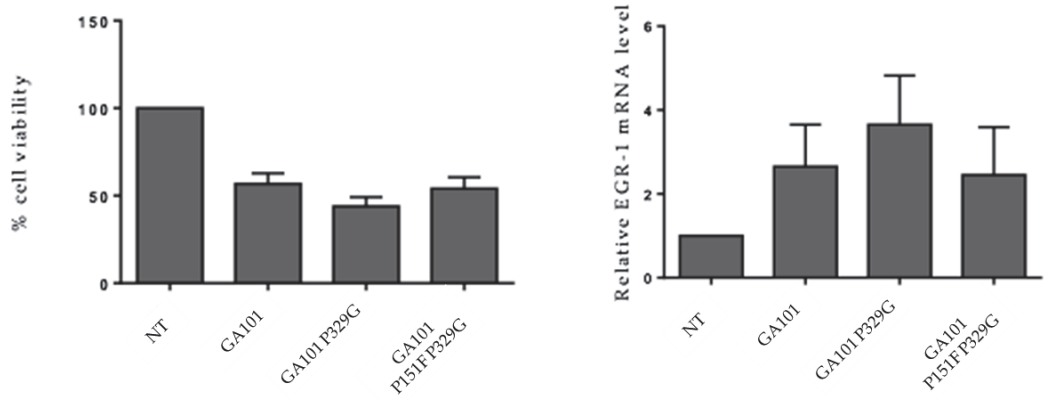
Supplementary Figure 2.



Supplementary figure 2. Anti-CD20 mAbs up-regulate EGR-1 expression *in vivo.* EGR-1 protein expression was evaluated in **(A)** Granta tumors overexpressing EGR-1 as well as **(B)** Namalwa tumors with knocked down EGR-1 expression. Tumors were collected 6 hours after GA101 treatment.
EGR-1 expression shown is from one representative tumor.

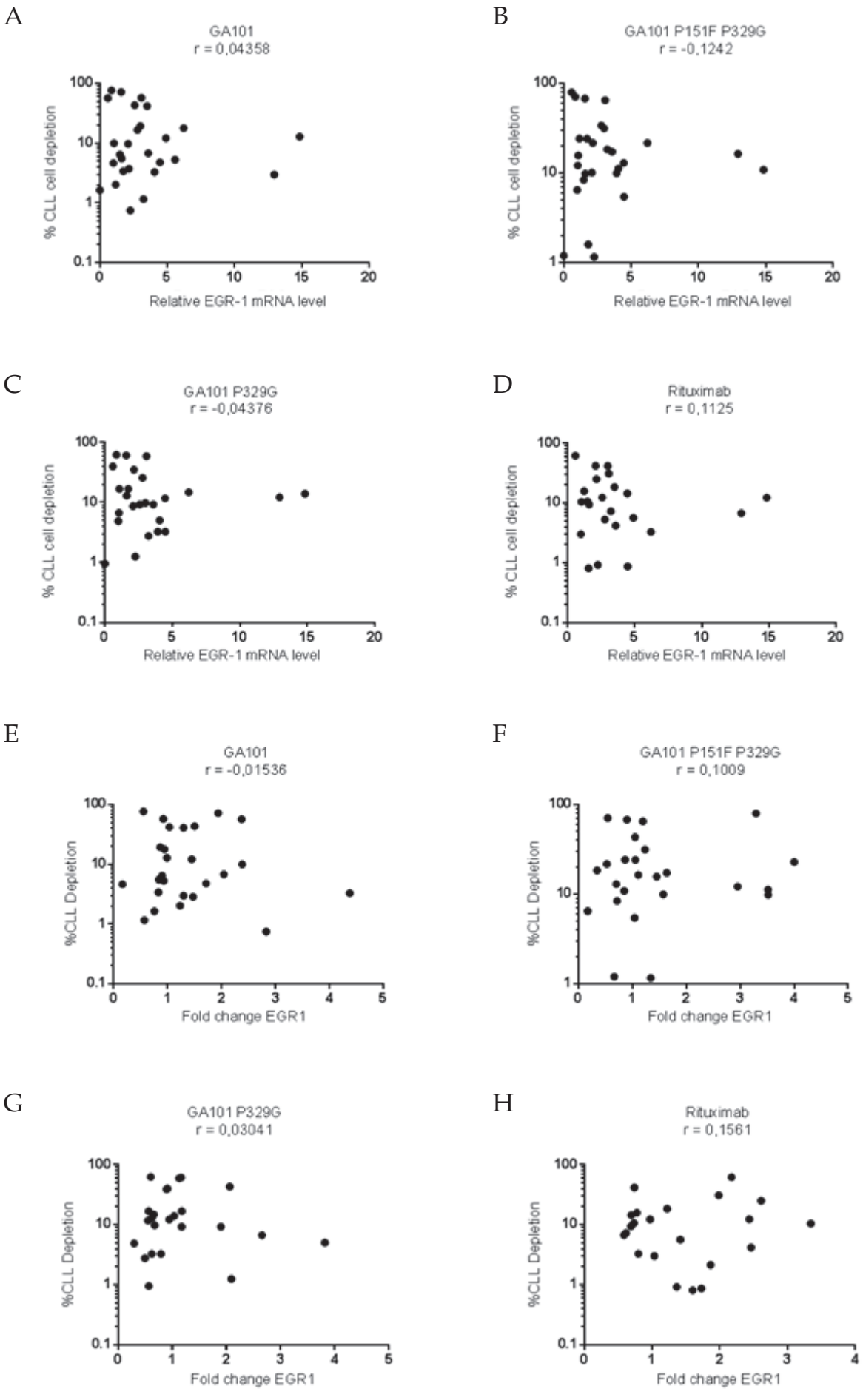
Supplementary Figure 3.

A. **B.**



Supplementary figure 3. GA101 variants up-regulate EGR-1 expression and induce cell death in human NHL cells. Analysis of EGR-1 mRNA expression by qRT-PCR in Granta cells following 3h exposure to 10 µg/ml anti-CD20 mAbs. EGR-1 mRNA level is normalized to the expression of the housekeeping gene 28S. (C) Cell viability evaluated by annexinV/PI staining on flow cytometry following 24h exposure to 10 µg/ml anti-CD20 mAbs. The cell viability was normalized with control untreated cells at 100%. Values shown are means +- SD of the least 3 independent experiments.

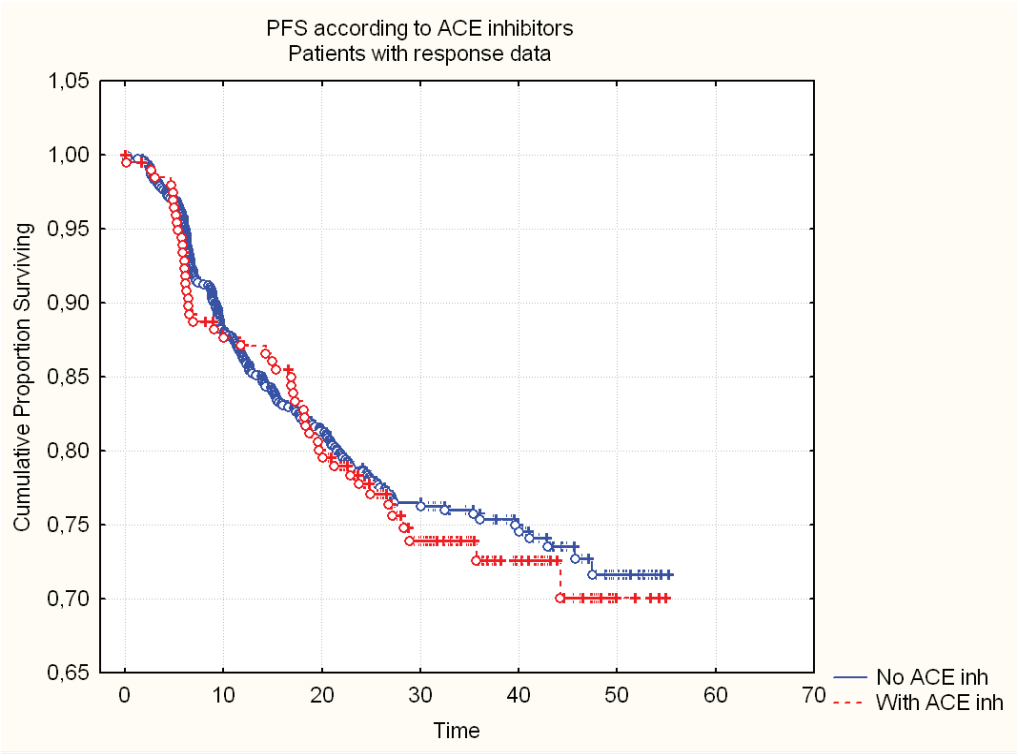
Supplementary Figure 4.



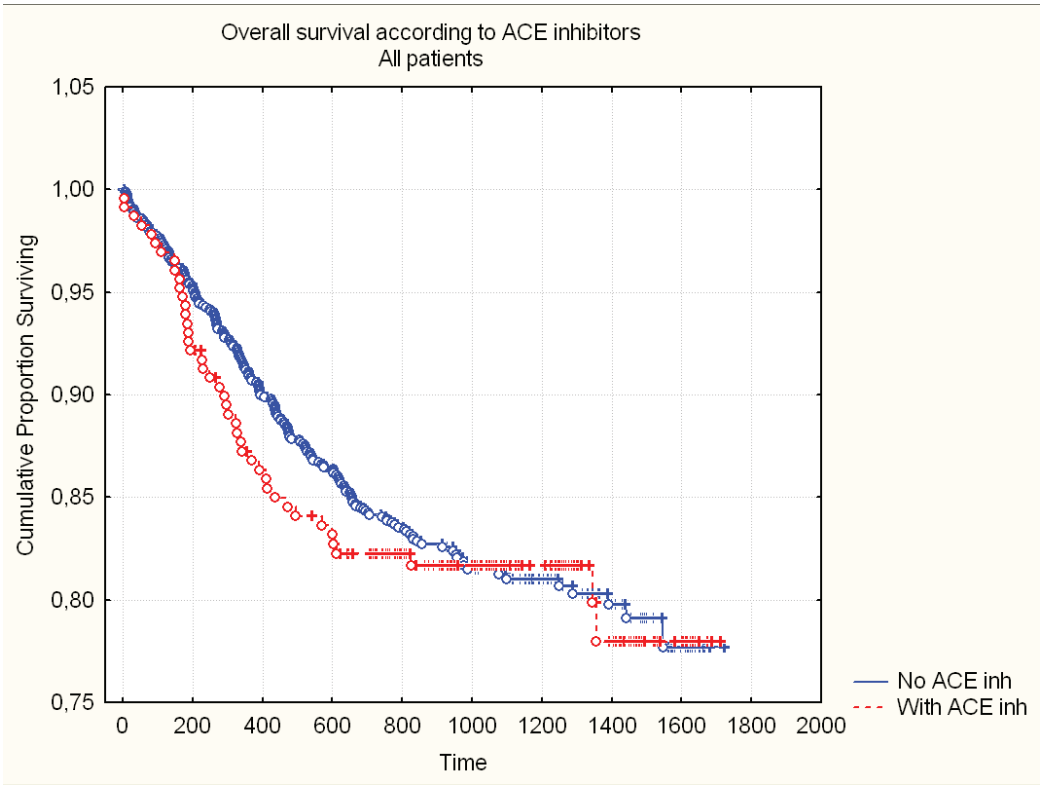
Supplementary Figure 4. EGR-1 expression does not correlate with the CLL depletion induced by anti-CD20 antibodies in CLL blood samples *ex-vivo.* Correlations between baseline mRNA expression of EGR-1 and CLL depletion induced by (A) GA101 (B) GA101 P151F P329G (C) GA101 P329G and (D) rituximab were analyzed using Spearman's rank correlation coefficient. Correlations between EGR-1 induction and CLL depletion induced by (E) GA101 (F) GA101 P151F P329G (G) GA101 P329G and (H) rituximab were analyzed using Spearman's rank correlation coefficient.

Supplementary Figure 5.

A.



B.



Supplementary figure 5. ACE inhibitors do not interfere with the efficacy of anti-CD20 mAbs therapy. Results from GOYA, an open-label, randomized, parallel group study evaluating the efficacy and safety of GA101 in combination with cyclophosphamide, doxorubicin, vincristine, and prednisolone or prednisone (CHOP) chemotherapy versus rituximab (MabThera/Rituxan) with CHOP in previously untreated participants with cluster of differentiation 20 (CD20)-positive diffuse large B-cell lymphoma (DLBCL). **(A)** Progression free survival curves and **(B)** overall survival of patients treated with rituximab and receiving or not ACE inhibitor.

CONCLUSION AND PERSPECTIVES

The results from our study have shown that anti-CD20 mAbs induce calcium influx, and the consequent increase in intracellular calcium content results in enhanced EGR-1 expression.

EGR-1 acts as a key factor in mediating the cell death induced by anti-CD20 mAbs, as its expression is required for the cytotoxic effect of anti-CD20 mAbs. However, the role of EGR-1 in response to immunotherapy has not been described so far. Further investigations would be necessary to fully describe its role and the signaling pathways regulated by EGR-1 in response to anti-CD20 mAbs. This could be of great importance as it would be interesting to investigate the possibility to pharmacologically modulate EGR-1 expression and the regulated pathways in order to enhance the cytotoxicity of anti-CD20 mAbs. We also propose that EGR-1 could be a potential biomarker to predict the response to anti-CD20 mAbs. In order to investigate this, a study is currently being performed in the Pathology Department in Lyon Sud Hospital, in collaboration with physicians working on follicular lymphoma. Our aim is to determine whether there is a correlation between the EGR-1 expression in a patient's sample and his response to therapy with an anti-CD20 antibody. The very first results from this study indicate an important variation of EGR-1 expression in patients as shown in figure 10.

We have shown that anti-CD20 mAbs induce calcium influx, which is crucial for the direct cytotoxic effect of anti-CD20 mAbs. Blocking the calcium flux with CCB impaired their antitumor efficacy in a preclinical model and was associated with worse outcome in patients diagnosed with diffuse large B-cell lymphoma (GOYA study). As CCB are widely used in clinic for the treatment of cardiovascular diseases, this observation is of potential clinical importance, as it raises the issue of the combination of anti-CD20 antibodies with CCB in patients. It also opens new directions for investigation. Firstly, further investigations are necessary to investigate the complex of calcium channels

A. B.

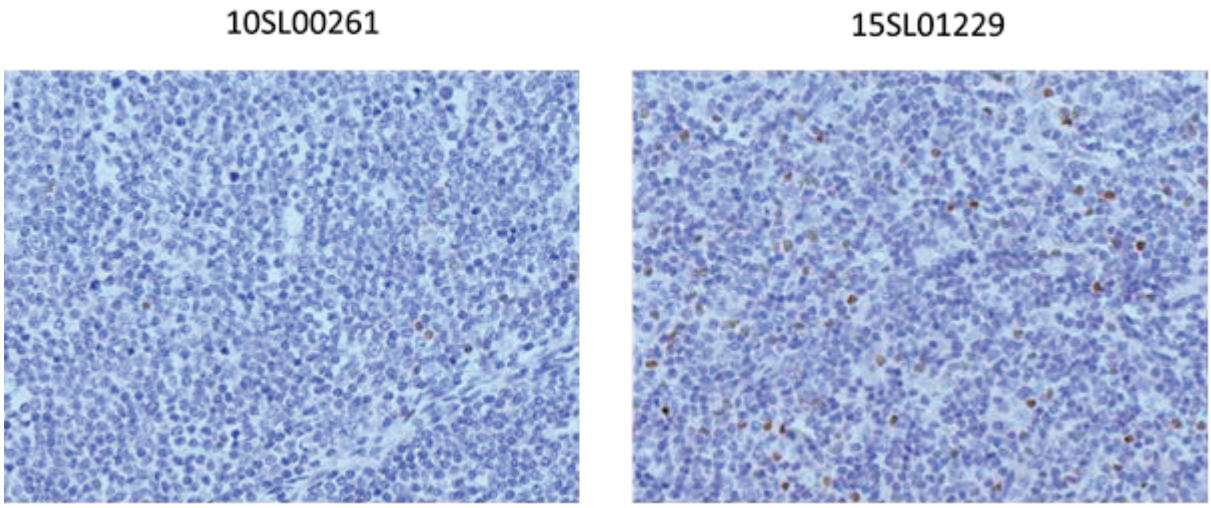


Figure 10. EGR-1 in human follicular lymphoma

EGR-1 was detected in immersion fixed paraffin-embedded sections of human follicular lymphoma tissue from two patients. In figure A is shown a section from patient with low expression of EGR-1 while in figure B is shown a strong EGR-1 expression in a second patient.

involved in anti-CD20 mAbs mediated calcium flux. This is important as rituximab and GA101 seem to differently induce calcium flux: rituximab induces capacitive and GA101 constitutive calcium entry. Additionally, we have shown that CCB specific for L-type calcium channels inhibit the calcium flux induced by anti-CD20 mAbs. As non-malignant B-cell do not express L-type calcium channels, it is crucial to investigate the expression and the role of these channels in lymphoma cells. This could lead to a better understanding of the role of calcium signaling in the biology of B cell lymphoma. Moreover, given the fact that calcium flux is required for the direct cytotoxic effect of anti-CD20 mAbs, it would be interesting to investigate the possibility to use calcium agonists to potentiate their anti-tumor activity.

In conclusion, our study provides a better understanding of the direct mechanism of action of anti-CD20 antibodies, and could result in a better use and enhanced efficacy of anti-CD20 monoclonal antibodies allowing patients to fully benefit from their therapeutic potential.

DISCUSSION

EGR-1 as a key factor in the direct cytotoxicity of anti-CD20 mAbs

The therapeutic landscape for B-cell malignancies has changed markedly two decades ago, when immunotherapy joined chemotherapy and radiotherapy as an effective approach for the treatment of cancer. Today, the anti-CD20 monoclonal antibodies are used to treat almost all types of non-Hodgkin's B-cell lymphomas. Anti-CD20 mAbs act by engaging Fc receptors on immune effector cells, such as NK cells and macrophages, and mediating antibody-dependent cell-mediated cytotoxicity/phagocytosis and complement-dependent cytotoxicity, in addition to inducing direct cell death. While the immune effector mechanisms of anti-CD20 antibodies have been widely studied and clinically proven, the direct cytotoxic effect of anti-CD20 antibodies is still widely uncharacterized.

In a previous preclinical study comparing the mechanism of action of rituximab and GA101, transcriptome analysis have evidenced EGR-1 significantly overexpressed after exposure both to rituximab and to GA101, suggesting that they may be involved in CD20-mediated signaling. [249] In the present study, we have shown that EGR-1 acts as a key factor in the direct cell death mediated by anti-CD20 antibodies. Using NHL cell lines and cell exogenously expressing CD20, we showed that the induction of EGR-1 is due to the activation of CD20 signaling. To further investigate the role of EGR-1 in the antitumor activity of anti-CD20 antibodies, we developed cell models in which we modulated EGR-1 expression. Decreasing EGR-1 expression by shRNA abolished the direct cytotoxic effect both *in vitro* and *in vivo*, indicating that EGR-1 is required for CD20-mediated apoptosis. The importance of EGR-1 in CD20 mediated apoptosis was confirmed by rescuing EGR-1 expression in EGR-1 knocked-down cells, which restored sensitivity to anti-CD20 mAbs. Additionally, the overexpression of EGR-1 resulted in enhanced cytotoxic activity of anti CD20-antibodies, confirming that EGR-1 acts as a key factor in the direct antitumor activity of anti-CD20 antibodies.

As an immediate early gene, EGR-1 is in the first-line of response of cells to different stimuli. Several studies identify EGR-1 as a major player in the response of cancer cells to cancer therapies. *Lasham et al.* demonstrated that EGR-1 levels are associated with the response to paclitaxel in triple negative breast cancer. [250] A reduced EGR-1 expression was associated with resistance to paclitaxel, while the up-regulation of EGR-1 increased the sensitivity to paclitaxel. Furthermore, *Kumar et al.* suggest that EGR-1 could be a candidate biomarker to predict response to anti-EGFR therapy with cetuximab, as EGR-1 was strongly induced in resistant cells. [251] In our study, we showed that the up-regulation of EGR-1 by anti-CD20 antibodies is associated with cell death induction. EGR-1 is involved in cell death regulation by direct transcriptional activation of pro-apoptotic genes. [140,145,252] Preliminary results have shown in response to anti-CD20 mAbs, EGR-1 might mediate cell death through the regulation of ATF3 and PTEN (data not shown). In fact, besides EGR-1, transcriptomic analysis have also revealed ATF3 strongly induced in B cells exposed to anti-CD20 antibodies. Moreover, we performed a screening of EGR-1 expression in different cell line, and we found a strong correlation between EGR-1 and ATF3 expression level. Additionally, the modulation of EGR-1 expression resulted in modulated ATF3 expression (data not shown). *Withlock et al*. have shown that in response to resveratrol treatment, EGR-1 associates with Krüppel-like factor 4 (KLF4) to induce ATF3 expression. [253] Further studies would be necessary to confirm a direct binding of EGR-1 to ATF3 promotor in our model. Another potential target of EGR-1 is PTEN. In addition to the overexpression of EGR-1, we also detected an increase in its sumoylated form. In a recently published paper the existence of a sumoylated form of EGR-1 was demonstrated. [145] Authors indicated that this post-translational modification is dependent on the phosphorylation of EGR-1 by Akt, which promotes its interaction with small ubiquitin related modifier (SUMO-1). Authors also showed that the sumoylated form of EGR-1 is a direct transcriptional activator of PTEN. In our model of exposure to anti-CD20 antibodies, we also found induction of PTEN expression (data not shown). PTEN is known to negatively regulate Akt pathway, and thus promotes apoptosis through the up-regulation of pro-apoptotic Bcl-2 family members. Further studies are necessary to elucidate the

EGR-1 regulatory network promoting cell death. A better understanding of this network of tumor suppressor genes, could be of great importance for a targeted cancer therapy. Finally, a further investigation of the importance of the sumoylated form of EGR-1 would be necessary. SUMO-1 is known to be involved in the modulation of proteins localization and function. [254] We did observe in CD20+ cells nuclear translocation of EGR-1 following the exposure to rituximab and GA101, which did not occur in CD20- cells (data not shown). It is likely that the sumoylated form of EGR-1 id necessary for its nuclear localization. Another hypothesis is that the sumoylation form of EGR-1 might be involved in the regulation of gene expression. Sumoylation of a transcription factor could affect its binding to DNA promoting either its association or dissociation from specific promoters. [255] Alternatively, sumoylation could promote or inhibit protein-protein interactions leading to differential recruitment of activities that might activate or repress gene transcription. Therefore, the sumoylation of EGR-1 could be of a great importance for its regulation and for directing EGR-1 in a pro-apoptotic pathway.

EGR-1 as a candidate biomarker to predict response to anti-CD20 therapy

Our results indicate that EGR-1 plays a crucial role in the direct cell death induction by anti-CD20 antibodies. Inhibition of EGR-1 induction results in absence of cytotoxic effect both *in vitro* and *in vivo*. Therefore, we investigated if we could correlate EGR-1 expression with B-cell depletion in blood samples from CLL patient. Although in the majority of the patients, the anti-CD20 mAbs induced EGR-1 expression, we did not find any correlation between EGR-1 expression level and the efficacy of anti-CD20 mAbs *ex-vivo*. This might be due to the fact that most of the patients were already treated for their disease. Therefore, a further study would be necessary to investigate the expression and induction of EGR-1 at diagnostic and a possible correlation with patient's outcome. In addition, we observed a strong variation in EGR-1 baseline expression among patients, which also did correlate with the efficacy

of anti-CD20 mAbs. A recent study by *Krysiak at al*. showed that EGR-1 is recurrently mutated in patients with follicular lymphoma. [256] Hence, it would be interesting to investigate if this is also the case in chronic lymphocytic leukemia, and if the mutational status of EGR-1 would interfere with the response to anti-CD20 mAbs.

The role of calcium influx in the anti-tumor activity of anti-CD20 antibodies

In our study, we have shown that both rituximab and GA101 were able to induce calcium influx upon binding to CD20. Although, the function of CD20 has not been fully elucidated, there is considerable evidence for its involvement in calcium transport across the membrane of B cells. It has been previously described that the binding of rituximab, induces aggregation of CD20 in the lipid raft, and an entry of extracellular calcium. [257] GA101, on the other hand, as a type II monoclonal antibody is believed to not be involved in the induction of calcium influx. We are the first to show that GA101 is also able to induce a calcium influx upon binding to CD20. Nevertheless, it seems that rituximab and GA101 do not induce the calcium influx through the same mechanism: rituximab induces capacitive calcium influx, while GA101 induces constative calcium influx. Out study has highlighted the importance of the calcium influx for the antitumorale activity of anti-CD20 mAbs and confirm that CD20 could function as a calcium channel or regulate the calcium flux. A further investigation would be necessary to fully elucidate the mechanism of induction and the function of CD20, as well the consequent induction of cell death. This would be of a great importance to fully seize the therapeutic potential of anti-CD20 antibodies and CD20 as a therapeutic target.

Calcium channel blockers impair the antitumorale activity of anti-CD20 antibodies

Calcium channel blockers represent a chemically and pharmacologically diverse group of agents that are widely used for the treatment of hypertension and angina. As the calcium signaling can promote apoptosis, several investigators have hypothesized that CCB use is associated with an increased risk for abnormal cell proliferation and tumor growth, by reducing the levels of intracellular Ca^{2+}. [258,259] In our study we have shown that calcium channel blockers impair the direct cell death induction by GA101 both *in vivo* and *ex-vivo* in blood samples from patients with CLL. We demonstrated that calcium channel blockers, antagonists of L-type voltage dependent calcium channel, inhibit the cytotoxic effect of anti-CD20 antibodies. L-type voltage dependent calcium channels are normally expressed in the membrane of excitable cells such as muscles and neurons. [260–262] Nevertheless, several studies indicate that the expression of L-type calcium channel could be found in cancer cells. *Chen et al*. have shown that L-type calcium channels are also overexpressed in prostate cancer and it has a functional role in androgen receptor transactivation. [263] In another study, *Zawadzaki et al*. demonstrated that human colon cancer cells express L-type calcium channels that mediate calcium influx and apoptosis. Moreover, the authors showed that verapamil inhibited L-type calcium channel mediated apoptosis in human colon cancer cells, and more importantly treatment with calcium channel blockers have been associated with increased colon cancer mortality. [246] This indicates that CCB could have a negative impact non only on the efficacy of anti-CD20 mAbs in B-cell malignancies, but also in other cancer types. However, so far no study has investigated the presence of L-type voltage dependent calcium channel in malignant lymphoma cells. As we have shown that malignant B-cells are sensitive to CCB, it is of great importance to investigate the presence of these channels.

More importantly, in this study we have demonstrated that patients receiving CCB and anti-CD20 therapy had a worst progression free survival and overall survival. This finding is of a great importance, as approximately 20% of the patients involved in the clinical trial also received CCB. Additionally, CCB are

often used in elderly patients, and the median age of CLL diagnosis ranges from 70 to 72 years, meaning that most of the patients diagnosed with CLL are likely to receive CCB treatment for cardiovascular disease. Thus, our results highlight the importance of coordinating drug administration in order for patients to fully benefit the therapeutic potential of anti-CD20 immunotherapy.

CONCLUSION AND PERSPECTIVES

In conclusion, our study has demonstrated that anti-CD20 induce calcium influx, and the consequent increase in intracellular calcium content results in enhanced EGR-1 expression. EGR-1 acts as a key factor in mediating the cell death induced by anti-CD20 mAbs. Furthermore, we have shown that the calcium influx and EGR-1 induction are required for the antitumorale activity of anti-CD20 mAbs, as blocking the calcium flux with CCB impaired their cytotoxic effect. More importantly, we have shown that CCB negatively impact the efficacy of anti-CD20mAbs in patients, and the subjects co-treated with CCB and anti-CD20 mAbs had a worst PFS and OS.

Several perspectives are possible for our study. As EGR-1 plays a crucial role, a further study would be necessary to confirm if EGR-1 could be a candidate as a biomarker to predict the response to anti-CD20 therapy. The role of EGR-1 in response to immunotherapy has not been describe and it is important to fully describe its role and the tumor suppressor network it is regulating. Additionally, it would be interesting to investigate the possibility to pharmacologically modulate EGR-1 expression and the regulated pathways in order to enhance the cytotoxicity of anti-CD20 mAbs.

In our study, we have shown for the first time that GA101 as a type II antibody is also able to induce calcium influx. Moreover, this observation strengthens previous ideas that CD20 is directly involved in the regulation of calcium flux across the plasma membrane. In addition, this opens new perspective for CD20 as a target for B-cell malignancies treatment. As we have shown that the calcium flux is necessary for cell death induction of anti-CD20 antibodies, it would be interesting to investigate the possibility to use calcium agonist to potentiate their anti-tumor activity.

Finally, the most important perspective and clinical relevance of our study, is the better understanding of the direct mechanism of action of anti-CD20 antibodies, and their interference with other medications, the CCB. Our study can have a direct clinical impact, as a better coordination of CCB and anti-

CD20 therapy, would allow patients to fully benefit the therapeutic potential of anti-CD20 monoclonal antibodies.

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