UNIVERSITÀ DEGLI STUDI
DI GENOVA

Scuola di Dottorato in Biotecnologie in Medicina Traslazionale

XXXIII Ciclo

Doctoral School of Biotechnologies in Translational Medicine

XXXIII Cycle

Expression of the IL12 and IL23 receptors and cytokines in Chronic Lymphocytic Leukemia and normal B cells

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Academic year: 2020/2021

Contents

Abstract **3**
Introduction **4**
Chronic lymphocytic leukaemia **4**
Epidemiology and Incidence 4
Diagnosis 4
CLL staging 5
Prognostic markers 6
Biology of CLL and microenvironment 8
CLL treatment 10
ODN and Toll like receptor 13
Cytokines and CLL 14
Aim of the study **16**
Materials and methods **17**
CLL cells samples 17
CLL cells isolation 17
CLL cells in vitro culture 17
Detection of Cytokines Receptors in CLL by Flow Cytometry 18
Normal B cells 18
Normal B cells in vitro culture 19
Detection of Cytokines Receptors in Normal B cell subpopulations by Flow Cytometry 19
Detection of IL23 and IL12 in CLL cultures 20
Immunoblotting 20
Statistics 21
Results **22**
CpG and CpG+IL-15 stimulations induce the expression of IL23R complex and IL12R complex in CLL cells 22
Proliferating CLL cells are those that express the IL23R and IL12Rß1 receptor chains the most 25
IL23R and IL12R chains expression is heterogeneous in CLL fractions 26
Expression of IL23R and IL12R in normal B cells 28
IL23 and IL12 secretion from CLL and normal B cells after stimulations 32
CLLs activated can signal through the JAK-STAT pathway 34
Discussion **36**
Activation of IL23R/I23 loop in CLL cells upon stimulation with CpG 36
Expression of IL12R/IL12 in CLL cells upon stimulation with CpG 37
Expression of IL23R and IL12R complexes in PB normal B cells 37
Expression of IL23 and IL12 receptor chains in CLL cell fractions 38
Ability of IL12R and IL23R complexes to signal in CLL cells stimulated with CpG 38
Bibliography **40**

Abstract

The mechanisms of clonal expansion of CLL are only partially understood. Several interactions of neoplastic cells with accessory cells and cytokines potentially sustaining neoplastic B cell clone survival and proliferation have been described. Recently, a paracrine/autocrine loop has been reported, involving the upregulation of the IL23R complex and IL23 secretion by CLL cells. This loop drives CLL cell clonal expansion in vitro and in xenografted NSG mice. Furthermore, in situ observations on tissue sections demonstrate that infiltrating IL23 secreting CLL cells interact with macrophages and CD40L expressing T cells. Although inducible in vitro by co-culturing CLL cells with T cells or CD40L expressing cells, the IL23 loop is not observed following stimulation of CLL cells via surface Ig or contact with nurse like cells or bone marrow stromal cells.

In this study, we investigated whether the IL23 loop could be induced following Toll-like receptor 9 (TLR9) engagement which influences leukemic cell survival, activation proliferation albeit in a heterogeneous manner. In addition, we explored the possible existence of an autocrine/paracrine loop mediated by IL12 which shares similarities and surface receptors with IL23 although with a likely opposite outcome in term of the possibility to sustain leukemic cell growth .

IL23R and IL12R complexes (IL23R/IL12Rβ1, IL12β2/IL12Rβ1) expression were evaluated by flow-cytometry following stimulation with CpG oligodeoxynucleotide (ODN) that binds the TLR9 on CLL, showing that CLL cells are able to express the IL23R complex on membrane and, at lower extent, the IL12R complex.

These receptors were assessed also in normal B cells by flow cytometry after 72h of stimulation with CpG and CpG+IL15. In this setting, normal B cells were less capable of IL23R complex expression compared to CLL cells. A further striking difference observed was related to the limited expression of IL12Rß2 receptor chain in stimulated CLL cells compared to normal B cells.

Supernatants of CLL cells and normal B cells were both tested for the production of these cytokines after stimulation. The results showed a low level of IL23p19 secretion for both CLL cells and normal B cells, which is significant after CD40L stimulation (used as positive control), and a higher production of IL12p70 which is more pronounced in normal B cells compared to CLL. In another series of tests, CLL cells were stimulated with CpG for 72h, and subsequently exposed to IL12 or IL23. Exposure to IL12 and IL23 induced the

expression of pSTAT1 and pSTAT3. Collectively our data corroborate the notion that IL23R complex act as a pro-survival factor for CLL cells. In contrast, the restricted IL12R complex expression in CLL cells compared to normal B cells indicated that the suppression of the expression of this receptor may favor the survival of the leukemic clones. The possibility of a reciprocal competition of the shared receptor chains is discussed.

Introduction

Chronic lymphocytic leukaemia

Epidemiology and Incidence

Chronic lymphocytic leukemia (CLL) results from a monoclonal expansion of $CD5^+$ B-lymphocytes in the bone marrow (BM), peripheral blood (PB), and secondary lymphoid organs, in particular lymph nodes (LNs).[1,2]

CLL is a clinically heterogeneous disease, with some patients never requiring treatment, others needing so after several years, and others requiring it at diagnosis. This degree of clinical heterogeneity may in part be attributed not only to clone-intrinsic biological features, but also to clone-extrinsic events related to the microenvironment. Indeed, the diverse signals that CLL cells sense in the surrounding environment are increasingly recognized as determinants of clone fitness and progression. [3,4]

Genetic factors can contribute to the development of CLL; indeed CLL is the most common adult leukaemia in western countries, whereas it is less common in Asia and relatively rare in Japan and Korea, even among Japanese people who immigrate to western countries.[2]

Diagnosis

In the presence of ≥ 5000 monoclonal B cells per μl in PB, over a period of more than 3 months, the diagnosis of CLL is commonly established by immunophenotyping. CLL cells co-express B and T cell surface antigens: CD19, CD20, CD23 and CD5 respectively. Furthermore, leukaemic cells derived from the same clone express the same immunoglobulin heavy chain, either kappa or lambda.

Recently, it has been confirmed that a panel of CD19, CD5, CD23, κ, λ and CD20 is sufficient for the diagnosis of CLL. In borderline cases, markers such as ROR1, CD79b, CD43, CD200, CD10 or CD81 could help the diagnosis.[5]

In the presence of <5000/µl B cells in the peripheral blood with a surface phenotype of CLL (light chain restriction and expression of CD5 and of low CD79ß) and no other features of lymphoproliferative disorder, the diagnosis is of "monoclonal B-lymphocytosis" (MBL), a precursor state of chronic lymphocytic leukaemia. Subjects with MBL, like CLL patients, show an increased risk of secondary cancers, in particular skin cancer.[5]

CLL staging

The definition of the CLL stage is based on the natural history of the disease. According to the European Binet staging system (developed in 1981), CLL is divided into three stages (A, B and C).[6] This system is based on the number of areas involved. Patients in Binet stage A have 0 to 2 affected lymph node areas or organ enlargement with normal levels of haemoglobin and platelets. Binet stage B patients have peripheral and medullary lymphocytosis and 3 to 5 affected lymph node areas. Binet stage C patients have peripheral and medullary lymphocytosis with Hb <10 g/dl and/or thrombocytopenia (<100,000/mm^3).
In the USA, the most common clinical staging system used is the Rai staging system developed by Dr Kanti Rai in 1975.[7] This method is based on the concept that CLL is an accumulative pathology. The Rai staging system divides the CLL disease into five (0-IV) different stages that are described in the Kanti R. Rai et al. paper as follows:

- 0, lymphocytosis in blood as well as in bone marrow (absolute lymphocytes, 15,000/cu mm or more in blood, 40% or more lymphocytes in the marrow).
- I, lymphocytosis with enlarged lymph nodes.
- II, lymphocytosis with enlarged spleen or liver or both. Nodes may or may not be enlarged.
- III, lymphocytosis with anaemia. Nodes, spleen, or liver may or may not be enlarged.
- IV, lymphocytosis with thrombocytopenia. Anaemia and organomegaly may or may not be present.

Patients belonging to the stage Binet B or Rai stage I/II are considered as intermediate-risk groups while patients belonging to the stage Binet C or Rai stage III/IV are defined as being in the high-risk categories.
For the overall status of the patient and the disease progression these two staging systems are very helpful, but they are unfit for the prediction of disease evolution. On the contrary,

molecular prognostic markers are more useful for predicting patient survival, disease progression and resistance to therapy.

Prognostic markers

Clinical and biological prognostic factors help define the risk for disease progression in individual patients and for the development of personalized treatment strategies. The most important prognostic factors in addition to the Rai and Binet clinical staging systems are divided into:

- genetic parameters, including the cytogenetic aberrations, mutational status of IGHV gene[8] and gene mutations, in particular *TP53* [9];
- cell markers, including CD38 [10] and ZAP70[11];

These parameters allow the stratification of the patients into different risk categories.

By using predominantly fluorescence *in situ* hybridization (FISH), different types of genomic aberrations have been identified.

The most common cytogenetic abnormality (in about 50% of CLL patients) is the deletion of the 13q14 region and subsequent loss of miRNAs (miR-15a and miR-16-1), which initiates leukaemogenesis. Usually, patients with this deletion have a good prognosis, even if, recently, it has been shown that a large deletion of 13q is associated with poor prognosis[12,13]. The other most common genetic abnormalities are represented by:

- the deletion of chromosome 11q22-q23, which is found in 10–20% of cases, causes the loss of the ataxia telangiectasia mutated (ATM) gene that encodes a DNA damage response kinase, ATM, that is very important in DNA damage detection and induction of cell cycle arrest. This alteration defines a subgroup of patients with unfavourable prognosis[13];
- trisomy 12, its prognostic relevance is still debate[14];
- deletion of chromosome 17p13 (del (17p13)), which occurs in 5–8% of cases, causes the loss of the tumour suppressor gene p53 (TP53) that is involved in the DNA damage detection pathway, cell cycle arrest, apoptosis and regulation of cellular metabolism[15]. Commonly, this type of alteration is acquired at the late stages of the disease particularly after treatment. Deletion of 17p13 is associated with poor survival and high risk of resistance to chemotherapeutic agents[16]. Therefore, patients that harbor this aberration are included in the highest risk prognostic category[17].

The somatic hypermutation status of the IGHV gene has a very important prognostic role. B lymphocytes are known to recombine immunoglobulin variable (V), diversity (D), and junction (J) genes during their development, to create a unique antigen binding domain of the B cell receptor (BCR). In order to generate high-affinity antibodies, a second process namely somatic hypermutation occurs. This process is triggered when the Ig binds the antigen and consists of the accumulation of point mutations in the V regions.

The presence or not of somatic mutations in the *IGHV* gene of leukemic cells divides CLL patients into two groups: unmutated CLL (U-CLL), with a germline identity ≥98%, who have an aggressive disease course, and mutated CLL (M-CLL) with a germline identity <98% who usually have a more indolent disease[8,18].

Somatic mutations can occur in the tumor suppressor gene p53 leading to a deterioration in its function[9]. It is well known that p53 exerts its tumor suppressive activities by inducing cell cycle arrest and apoptosis[9,19].

Patients harbouring these mutations are characterized by an aggressive disease course and resistance to chemotherapy and chemoimmunotherapy, as is well documented by a variety of studies[9,16,20], including prospective clinical trials[21,22]. It has been shown that in about 5% of untreated patients, TP53 mutations take place in the absence of del (17p13) while in about 70% of cases, TP53 mutations can occur together with del (17p13) leading to a double mechanism of inactivation[9]. It has to be highlighted that TP53 represents an independent adverse prognostic marker for progression free-survival (PFS) and overall survival (OS) in CLL patients[9].

Recently, thanks to the use of next generation sequencing, new genomic abnormalities have been identified, for example i) activating mutations of *NOTCH1*, which occur in about 10% of CLL patients at diagnosis and are associated with poor prognosis, poorer responses to conventional chemoimmunotherapy and increase in the risk of transformation to diffuse large B-cell lymphoma (DLBCL)[23]; and ii) dysfunctional mutations of Splicing factor 3b, subunit 1 (*SF3B1*), which occur in about 5%-15% of patients with CLL at diagnosis and are related to poor overall survival, faster disease progression and chemoresistance[24].

CD38 is a transmembrane glycoprotein that is commonly expressed at high levels in plasma cells, germinal centres B cells, B cell precursors and, with low expression, on circulating B cells[10]. The natural ligand of CD38 is CD31, an adhesion molecule expressed by different

cell types in the CLL microenvironment, such as "nurse like cells" (NLCs) and T lymphocytes. The binding between CD38 and CD31 leads to survival and proliferation signals in leukaemic cells[10]. CD38 is defined as a negative prognostic marker for patients with CLL[10]. In the majority of studies, the threshold, that provides the best prognostic value, is $CD38^{+} \geq 30\%$ (Z., 2005).
Like CD38, ZAP-70, a member of the syk tyrosine kinase family, is a negative prognostic marker for patients with CLL[11].

Biology of CLL and microenvironment

CLL is characterized by the accumulation of CD5 and CD19 positive monoclonal B cells in the peripheral lymphoid organs, bone marrow and blood[1,25]. CLL cells are phenotypically similar to mature B cells that have encountered the antigen. Indeed, they express high levels of surface molecules (CD23, CD20 dim, CD69) and low levels of markers downregulated after cellular activation, such as CD22, receptor gamma Fc IIb and CD79b[1,18]. Furthermore, the expression levels of surface immunoglobulin, CD20, CD79b and CD19 are usually low compared with normal B cells. Yet, they express a typical marker of the memory B-cell, CD27[26] and also the gene expression profiles are similar.

In the past, CLL cells were considered to be quiescent apoptosis-resistant malignant B lymphocytes. This resistance was associated with the over-expression of the anti-apoptotic protein Bcl-2[27]. CLL was in fact described as a "pathology of accumulation", considering the defect in the apoptotic mechanism as one of the main causes of the disease. Over time, different studies on CLL biology revealed a new dynamic view of this disease[28]. It became clear that CLL cells undergo, during their life, iterative cycles of re-activation and subsequent clonal expansion, before coming back to a quiescence state. By using a deuterium oxide 2H in vivo labelling method, in which patients consumed deuterated (heavy) water ($2H_2O$), it has been shown that the daily growth rate of CLL cells ranges from 0.08% to 1.7%[29]. Furthermore, by using this same method, it has also been demonstrated that the lymph nodes are the anatomical site harbouring the largest fraction of newly born cells compared to peripheral blood and bone marrow[30]. This supports the concept that activation and clonal expansion occur in lymphoid proliferation centres within secondary lymphoid tissues, where multiple molecular interactions with antigen and microenvironment contribute to leukaemic B cell survival, chemoresistance and proliferation.

The tumor microenvironment (TME) is a very complex milieu in which signals, fundamental for the survival and accumulation of leukaemic cells, are delivered by soluble factors or direct cell contact. An important interaction that rescues CLL cells from apoptosis, both in vivo and in vitro, is represented by the binding between CD38, expressed on CLL cells, and its natural ligand CD31, expressed on NLCs[31] and T lymphocytes [1]. Bone marrow stromal cells (BMSC)[32], the predominant stromal cell population in the CLL microenvironment, can also protect CLL cells from apoptosis and support their proliferation. Crucial for supporting growth and survival of leukaemic cells is the binding between CD40, expressed on CLL cells, and CD40 ligand (CD40L), expressed on activated CD4+ T helper lymphocytes[1,33]. In addition, T cells secrete different types of cytokines, including IL4 and IL21, that play an important role in supporting CLL cells proliferation[1,33,34].

Another soluble factor that might support the clone expansion is represented by CXCL12 (stromal cell-derived factor 1, SDF-1), a natural ligand of the chemokine receptor CXCR4 that is overexpressed on CLL cells. CXCL12 is secreted by NLCs and, after binding CXCR4, promotes the up-regulation of anti-apoptotic genes, like Bcl-2 and Mcl-1, favoring the survival of leukaemic cells[1]. Also IL6, produced by endothelial cells, inhibits apoptosis of CLL cells[35].

All these results indicate a dynamic picture where CLL cells undergo iterative cycles of re-activation and subsequent clonal expansion in the lymphoid microenvironment, which crucially contribute to CLL disease progression.

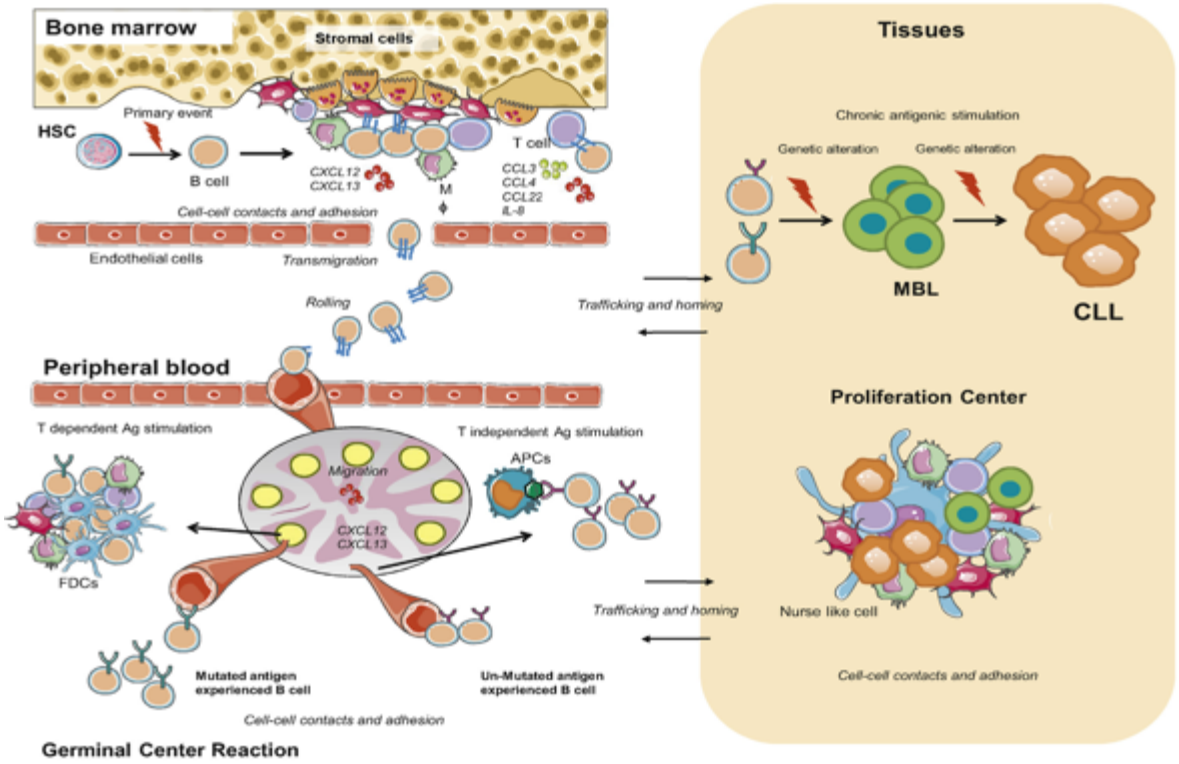


Fig. 1 Interaction between CLL cells and microenviroment

CLL treatment

Since CLL disease is still an incurable disease, its pharmacological treatment remains a subject of considerable interest and relevance. Patients with CLL have a variable disease course with a third of patients never needing treatment and, on the other hand, other patients that need treatment soon after diagnosis. The criteria for initiating treatment in CLL patients depend on the symptoms caused by the disease and also on the Binet/Rai staging systems. In particular, patients with asymptomatic, early-stage disease (Rai 0; Binet A) are usually not treated but are followed on a "watch and wait" principle[6,7,13]. On the contrary, patients with signs of active disease (i.e. rapidly progressive lymphadenopathy) or classified as Binet stage C or Rai stage III and IV, are subjected to treatment[6,7,13].

For several decades, the “gold standard” treatment for CLL was represented by the alkylating agent chlorambucil[36]. It was discovered in the ‘90s that the combination of cyclophosphamide, another alkylating agent, and the purine analogues Fludarabine improved the quality and duration of response in younger patients[37].

Fludarabine (FAMP) is the most studied and effective purine analogue in CLL. The purine analogues act by mimicking the purine's structure and after their incorporation into DNA or RNA, they inhibit the cellular replication and transcription. Fludarabine is also able to induce cell death in quiescent cells by the activation of the mitochondrial pathway of the apoptotic cascade[38]. However, a considerable clinical problem is the development of chemoresistance. This might be due to genetic alterations, like *MYC* overexpression[39], del(17p)[16] and/or TP53mutations[16], but is also markedly influenced by the TME, which induces the expression of anti-apoptotic Bcl-2 family proteins[40], thus contributing to chemoresistance[41,42]. As an example, the Mcl-1/Bax ratio and also Bcl-2/Bax ratio was reported to correlate with chemoresistance to Fludarabine[41,43]. Also, the induction of both Bcl-xL and A1 in CLL was associated with chemoresistance to Fludarabine in preclinical models[44].

Drugs that target two fundamental kinases involved in the transduction of signals from the BCR, namely the Bruton's tyrosine kinase (BTK) and the phosphatidylinositol 3-kinase (PI3K) δ isoform, have been developed in recent years.

The BTK pathway is amplified in CLL, where this kinase is constitutively phosphorylated, and leads to pro-survival signals and induction of proliferation by its effect on AKT[45], nuclear factor-KB (NF-KB)[46] and extracellular signal-regulated kinase (ERK)[47].

PI3K is a family of enzymes involved in crucial cellular activities such as proliferation, survival, metabolism, migration and genomic instability[48,49]. PI3Kδ isoform expression is restricted to leukocytes and plays a central role in the survival of normal B cells. In CLL cells the PI3K pathway is constitutively activated and dependent on PI3Kδ[50]. Therefore, these two kinases represent a very good target for the CLL disease. Ibrutinib (PCI-32765), a covalent inhibitor of BTK, and Idelalisib (GS-1101 or CAL-101), a selective and reversible inhibitor of PI3Kδ, were particularly successful among drugs developed for inactivating these kinases. They are able to reduce, both *in vitro* and *in vivo,* cell survival, migration and proliferation[50-52] of CLL cells and are currently FDA approved for patients with relapsed or refractory CLL and also in patients with TP53 aberrations[53,54].

However, recent data report the development of CLL cells resistances to Ibrutinib[55,56]. Also, immune-mediated toxicity like neutropenia and sepsis, hepatotoxicity and pneumonitis have been reported in patients on Idelalisib[57].

Another drug that showed a remarkable clinical response in CLL, independently from negative prognostic markers like TP53 aberration[58], is the B-cell lymphoma 2 (Bcl-2) Homology 3 (*BH3*)-mimetic Venetoclax (ABT-199).

CLL cells rely on the activity of anti-apoptotic Bcl-2 family members for their survival[59]. Therefore, strategies to restore apoptosis in CLL cells by antagonizing the anti-apoptotic proteins have led to the development of BH3 mimetics as therapeutic agents[60-64]. *BH3-mimetics* are small molecules modelled on the BH3 domains of BH3-only members[62] (Baell JB, 2002). They mimic their function by binding to anti-apoptotic Bcl-2 family proteins, thus leading to the inhibition of their activity. In this way, Bax and Bak are released, causing the trigger of apoptosis[59,63].

The prototype of these small molecules was ABT-737, which mimics the pro-apoptotic protein Bad[64]. Its oral derivative is Navitoclax (ABT-263) that binds, with high affinity, the anti-apoptotic proteins Bcl-xL, Bcl-W and Bcl-2 but not Mcl-1[63]. It has shown promising results in haematological malignancies, especially in CLL[61]. However, ABT-263 provoked severe thrombocytopenia due to on-target toxicity on the Bcl-xL protein, which is a fundamental survival molecule for the platelets[63,65,66]. In order to avoid this important side effect, the second-generation compound ABT199 (called also Venetoclax) was designed for specifically binding Bcl-2[60,61] sparing thus Bcl-xL and platelets. In particular, ABT199 shows a subnanomolar affinity for Bcl-2, no interaction with Mcl-1 and very weak affinity for BcL-xL and Bcl-W[60]. This drug has been demonstrated to be efficacious in leukaemia/lymphoma Bcl-2 dependent cell lines and also in tumor xenograft models[60]. Its effect seems superior in CLL than in other lymphoid malignancies, although evidence in mantle cell lymphoma and lymphoplasmacytic lymphoma is encouraging[67]. In recent times, a phase I[61] and a phase II[58] studies have proved that Venetoclax monotherapy is active also in patients with relapsed or refractory del(17p). However, the cytotoxic activity of this BH3-only mimetic is impaired when anti-apoptotic proteins, like Mcl-1 and Bcl-xL, are overexpressed[68,69]. Recently, Tahir S.K. et al. have shown that in leukaemic and lymphoma cell lines resistant to ABT199 there is an increased expression of the anti-apoptotic proteins Mcl-1 and Bcl-xL, which are not targeted by ABT199[69]. The work of Thijssen R. et al. analysed in CLL cells to what extent microenvironmental signals can alter sensitivity to ABT-199[68]. They reported that unstimulated CLL cells are highly sensitive to ABT-199 (LC50<1nM) rather than CD40 and CD40+IL-4 stimulated CLL cells that show to be fully resistant to 10 μM ABT-199. This might be due to the over-expression, in the proliferating

CLL cells, of anti-apoptotic proteins that are not targeted by ABT199, like Mcl-1 and Bcl-xL[68].

For all these reasons, it appears that the microenvironment and subsequent CLL cells activation/proliferation reduce CLL cell sensitivity to Venetoclax.

The complexity of the cross-talk between CLL cells and the microenvironment, as well as the mechanisms of drug resistance and treatment failure need to be further investigated.

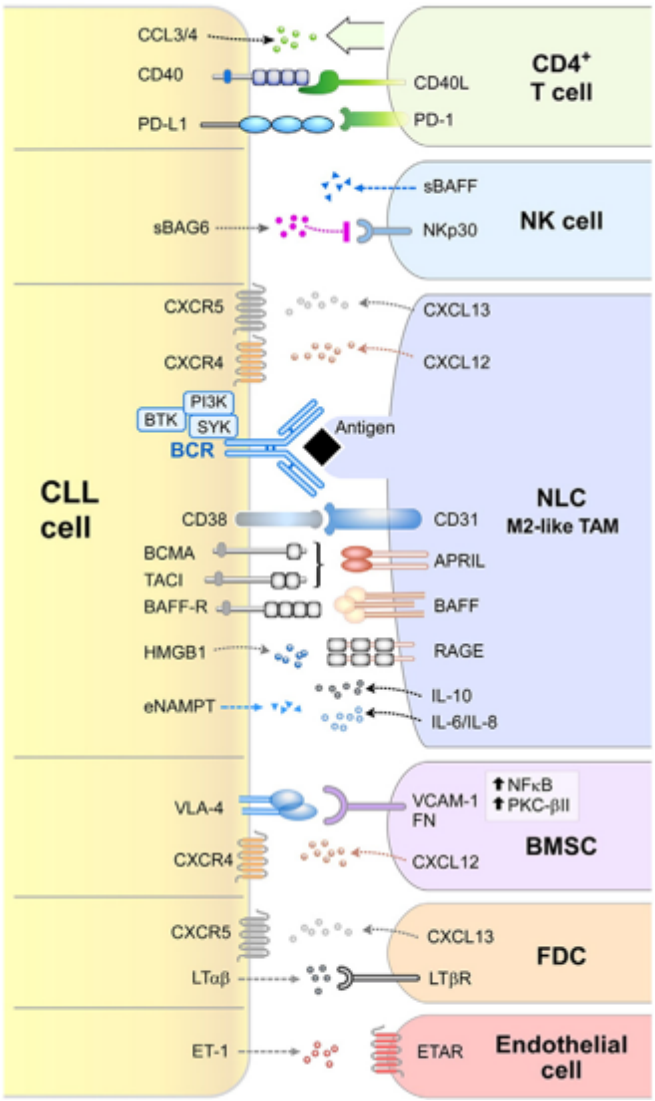


Fig.2 Molecules of the CLL microenvironment

ODN and Toll like receptor

CpG oligonucleotides (ODNs) are synthetic ODNs that contain unmethylated CpG dinucleotides in specific sequences. These ODNs have been seen to be recognized by TRL-9 with strong immunostimulatory effects.

Three types of CpG ODNs, type A, B and C, have been identified which differ in their immunostimulatory activities. Type A ODNs are characterized by a palindrome sequence and a 3 'poly-G sequence. They induce a high production of INF-α by dendritic cells but are weak stimulators of TRL-9.
Type B ODNs contain one or more CpG dinucleotides in their sequence. They strongly activate B cells, on the contrary they weakly activate the secretion of INF-α.
The latter, type C, combine the characteristics of both previous types in fact induce both the secretion of INF-α and the stimulation of B cells.
In fact, recent studies have shown that the exposure of B cells to ODN and IL15 (cytokine present in the bone marrow, lymph nodes and spleen) leads to a vigorous proliferation[70].

Toll like receptors are transmembrane structures usually react with exogenous and at times endogenous proteins, whereas the latter recognize nucleic acids of microbial or endogenous origin. TLR9 and TLR7 are expressed by normal and malignant B lymphocytes intracellularly in endosomes. Stimulation of both types of receptors influences B-cell survival and proliferation of normal B lymphocytes[71,72] and BCRs can have pathogenic roles in CLL[73] and B-cell lymphomas[74].
It has been shown that both the clone antigen receptor and the milieu stromal appear to influence the growth rate. Furthermore, the involvement of TLR signals probably seems to be based on a high atypical expression of TLR9 on the CLL B cell membrane and the likelihood that the specificity of BCR facilitates the internalization of physically bound molecules to CpG sites[70]. The high expression of TLR9 and the specificity of the BCR for DNA or DNA-bound antigens suggest that TLR9 signals are important in guiding the growth of the leukemic clone in patients[1,70].

Cytokines and CLL

Pro-inflammatory cytokines have been variously observed and used to identify subsets of CLL with a more aggressive course and worse survival[75,76]. These results suggest that inflammatory cytokines may provide a strong incentive for CLL origin and progression. Among the pro-inflammatory cytokines interleukin-23 is a heterodimeric cytokine composed of a p19 subunit and a p40 subunit, which is common to IL12. IL23 is predominantly produced by myeloid dendritic cells and type 1 macrophages in microbial

response or host immune stimuli, and is involved in the regulation of immune responses against infection and tumor development through IL receptor binding -23 (IL23R)[77,78].
IL12 is a pro-inflammatory cytokine as well, is composed of a p35 and p40 subunits and its receptor is made by IL12Rß1 chain and IL12Rß2 chain. This cytokine is generally produced by DCs, macrophages and B cells in response to microbial pathogens[79]. It can also induce the production of IFNγ by T cells and NK cells and induce the Th1 differentiation[79].

Regarding hematological tumors, IL23R heterodimer has been seen to be upregulated in primary acute lymphoblastic leukemia cells, follicular lymphoma, and diffuse large B cell lymphoma, where it inhibits the growth of neoplastic cells after cross linking with its ligand [80,81]. Interestingly, CLL patients have significantly higher serum IL23 levels than those of age-matched healthy donors, albeit with some heterogeneity among CLL patients[82], indicating a possible role in this disease.

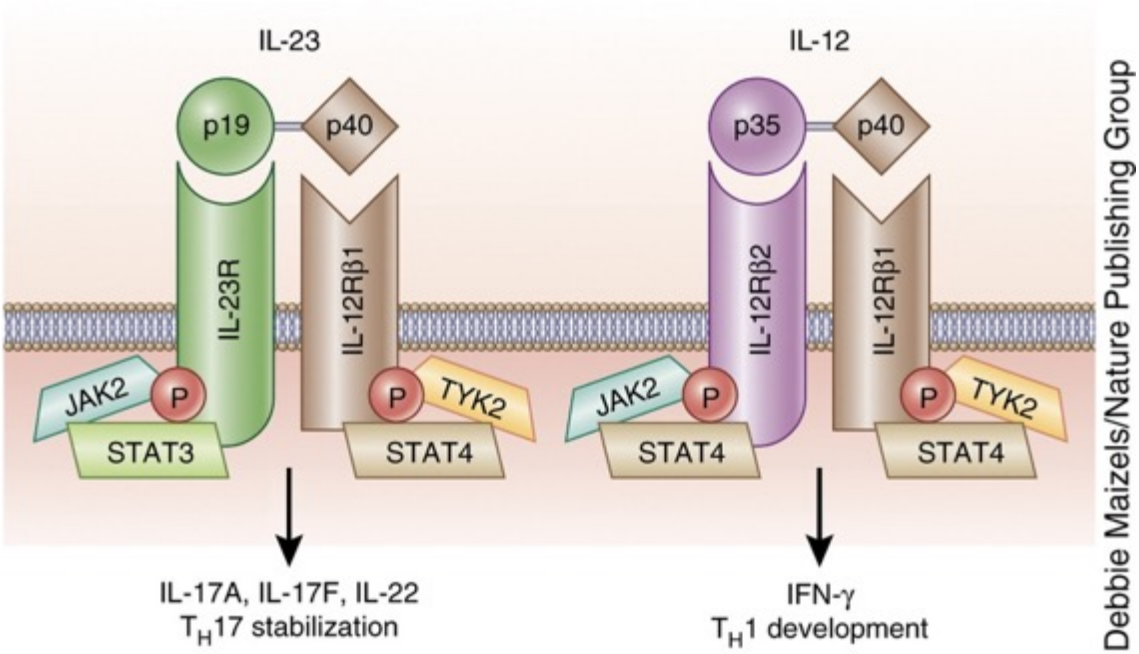


Fig. 3 Scheme of IL23 and IL12 cytokines

Aim of the study

A previous study[83], has shown that circulating CLL cells of a large prospective series of early stage, Binet A patients variably express the IL23R subunit in the absence of the IL12Rβ1 chain, and that the expression of the IL23R subunit is positively correlated with adverse prognostic factors and a higher risk of therapy need. In addition, T-dependent (CD40L) stimulation of CLL cells was shown as capable to induce the full expression of IL23R complex (IL23R/IL12ß1) and the secretion of IL23 by CLL cells. Thus, evidence of a stroma-induced regulation of the IL23R complex was provided and an autocrine/paracrine loop involving IL23R complex upregulation and IL23 synthesis in CLL clones functional to their fitness was unveiled. The trophic nature of the IL23/IL23R axis in CLL cells was demonstrated in vivo by treatment with an anti-IL23p19 monoclonal antibody (IL23p19), which eradicated xenografted CLL clones in the infiltrated tissues by effectively inhibiting proliferation and inducing apoptosis.

In this study, we investigated if the signaling through a different pathway was able to induce the IL23 loop. In particular, we focused on the stimulation of Toll-like receptor 9 (TLR9) which engagement influences leukemic cell survival, activation and proliferation in a heterogeneous manner.

In addition, to have a better comprehension of the CLL microenvironment, we studied another cytokine of the same family: the IL12 and its receptor (IL12R). This cytokine has a pro-inflammatory function as well and shares with the IL23 a receptor chain and a cytokine subunit but it may negatively impact on leukemic clone expansion.

Finally, to understand if the expression of these receptors and the production of their cytokines or if the activation of a possible loop represents an “ectopic” feature characterizing CLL cells we decided to check the status of these cytokines and their receptors also in resting and activated normal B cells.

Materials and methods

CLL cells samples

The study was approved by the Institutional Review Boards of Northwell Health and was conducted according to the principles of the World Medical Association Declaration of Helsinki. CLL patients were diagnosed as recommended[84], and all subjects provided written informed consent at enrollment.

CLL cells isolation

CLL cells from each patient's PB were isolated by negative selection using RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, BC). Whole PB was incubated with the mixture, then diluted with 2% FBS in PBS and centrifuged over RosetteSep DM-L Density Medium (Stemcell Technologies). CLL samples initially purified by this technique were tested for purity by the Center for CLL Research.
Cells were then resuspended in freezing solution and cryopreserved in liquid nitrogen. Samples from CLL patients that containing at least 95% of leukaemic cells were considered eligible for the study.

CLL cells in vitro culture

Cell cultures of CLL cells were performed by seeding thawed cells in an enriched medium used for normal B cell replication in long-term[85] cultures with added insulin/transferrin/selenium supplement (CAT #17-838Z; Lonza). Notably, this medium contains the reducing agent, 2-ME (5×10^{-5} M). The latter replaces an important function of bone marrow stromal cells in converting cystine to cysteine, which is needed for CLL uptake and use in the glutathione synthesis needed for retained viability[86]. Fresh medium was prepared for each experiment using stock addivities. Cultures were routinely established in 96-well round bottom plates at $4x10^5$ cells per 200-µl volume with duplicates for each culture condition. Recombinant human IL-15 (PeproTech Inc.) and CpG DNA TLR9 ligand (ODN-2006; Invivogen) were added at final culture concentrations of 15 ng/ml and 0.2 µM (1.5 µg/ml) for 72h respectively[70].

Detection of Cytokines Receptors in CLL by Flow Cytometry

Live cells were identified using LIVE/DEAD Fixable Stains for flow cytometry (LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit or Far Red Dead Cell Stain Kit, Life Technology). For surface membrane immunofluorescence, cells (2×10^5) in FACS buffer (PBS + 10% bovine serum albumin + 1% sodium azide) were incubated with primary antibody for 20 min at 4°C, followed by fixation with 0.1% formaldehyde in PBS.
The same procedure and number of cells were used for the isotype control.
To detect the different chains, the following mAbs were used: IL23R (cat #FAB140019-100, R&D Systems), IL12Rß1 (cat #565043, BD Horizon), IL12Rß2 (cat #FAB1959C, R&D Systems). Data were acquired with a BD LSR Fortessa flow cytometer using the HTS plate reader and analyzed by FlowJo 10.6.2 version.

CLL proliferation and activation were determined checking the level of EdU incorporation, Click-iT™ EdU Alexa Fluor™ 647 Imaging Kit (ThermoFisher Scientific) and CD86 mAb expression (cat #555657, BD Pharmigen).

Detection of Cytokines Receptors in CLL fractions by Flow Cytometry

PBMCs were thawed and stained for the surface markers CXCR4 APC (cat #306510, BioLegend) and CD5 PE-Cy7 (cat #300622, BioLegend) and CD19 Pacific Blue (cat #48-0199-42, ebioscience). For the staining, cells (2×10^5) in FACS buffer (PBS + 10% bovine serum albumin + 1% sodium azide) were incubated with primary antibody for 20 min at 4°C, followed by fixation with 0.1% formaldehyde in PBS.
This staining allowed us to study 3 different fractions: Proliferative fraction (PF) CXCX4 dim/CD5 bright; Resting fraction (RF) CXCR4 bright/CD5dim; Intermediate fraction (IF) CXCR4 dim/CD5dim[87]. For each of these fractions we checked the expression of the receptors described above.

Normal B cells

Normal B lymphocytes were obtained from the PB of healthy volunteers.
Mononuclear cells were isolated by Ficoll density gradient centrifugation as a pre-separation step. Normal B cells were isolated by negative selection using a B cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's

instructions. We performed a staining with CD19, CD5 and CD3 mAbs, to verify *B lymphocytes purity*.

Normal B cells in vitro culture

Cell cultures of normal B lymphocytes were performed by seeding purified cells into RPMI culture medium supplemented with 10% FBS at 2×10^6/ml in 24-well plates each containing 1 ml of culture medium.

In vitro activation of B cells was achieved by co-culturing B-lymphocytes with Recombinant human IL-15 (PeproTech Inc.) and CpG DNA TLR9 ligand (ODN-2006; Invivogen) at the final concentration of 10 ng/ml and 2,5 µg/ml for 72h.

Detection of Cytokines Receptors in Normal B cell subpopulations by Flow Cytometry

Live B lymphocytes were stained using 7-AAD (cat #51-68981E, BD), 1x10 cells in FACS buffer were incubated for 10 min in the dark.

To detect each subpopulation a flow cytometry panel with 9 different fluorochromes was built. We started our analysis with a dot plot IgD APC-Vio770 (cat #130-110-646, Miltenyi Biotech) vs CD27 PE-CF594 (cat #562297, BD) that allowed us to discriminate 4 subpopulations; *Naïve* (NA) CD27-/IgD+; *IgM Memory* (MM) CD27+/IgD+*; Switch Memory* (SM) CD27+/IgD- *and Double negative* (DN) CD27-/IgD-.

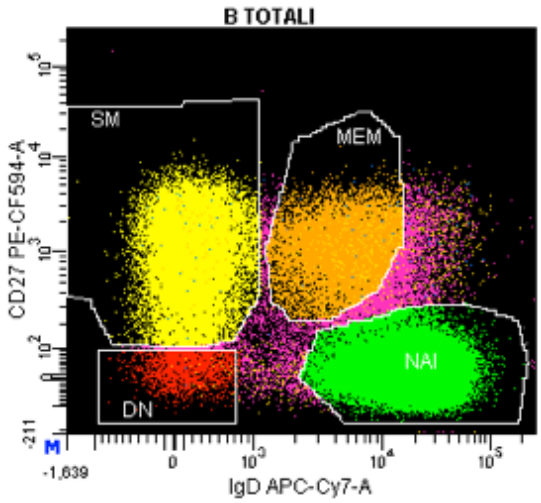


Fig. 4 Example of B subpopulations gating

For each subpopulation we investigated the expression of the single receptors chains using the following mAbs: IL23R PE (cat #FAB14001P-100, R&D Systems); IL12Rß1 FITC (cat #FAB839F, R&D Systems); IL12Rß2 APC (cat #130-125-974, Miltenyi Biotech).

Detection of IL23 and IL12 in CLL cultures

Supernatants were collected from CLL cells cultures after 72h and tested with the MILLIPLEX MAP Human High Sensivity T cell magnetic bead panel (cat #HSCTMAG-28SK-03, Merck Millipore) using the LUMINEX instrument.

Immunoblotting

CLL cells, either untreated or treated with CpG/CpG+IL-15 2,5 µg/ml for 48h, were washed with ice-cold PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and lysed in lysis buffer (20 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4 and *protease inhibitor* (Sigma Chemical Co., St Louis, MO, USA) for 20 min on ice. Lysates were centrifuged 10 minutes at 10.000 *g* and supernatants collected. Protein concentration was assessed by the Bradford method. Samples were denaturated by the addition of Laemmli sample buffer 4x and were boiled for 5 min. 30 µg of total proteins were used for each sample and loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). Run was performed at 4°C, at 50 mA for each gel, for 120 min. Electrophoretically separated samples were transferred onto nitrocellulose (NC) membranes by electroblotting, at 400 mA, at 4°C for 90 min. NC membranes were blocked: 1h in 1X PBS, 0.1% Tween® 20 with 5% w/v Bovine serum Albumine (BSA) for the phosphorylate proteins, and 1h in *1X* PBS, *0.1%* Tween® 20 with 5% w/v nonfat dry *milk* for the total proteins; then the membranes were incubated, over-night at 4°C, with the specific primary antibodies (Abs): anti Phospho-JAK2 (Tyr1008) (Cell Signaling Tech.), anti JAK2 (Cell Signaling Tech.), anti phospho STAT3 (Tyr705) (BD TRANDUCTION LABORATOIRES), anti STAT3(BD TRANDUCTION LABORATOIRES), anti phospho-STAT1 (Tyr701) (Cell Signaling Tech.), anti STAT1 (Cell Signaling Tech.), anti phospho-STAT4 (Cell Signaling Tech.), anti STAT4 (Tyr693) (Cell Signaling Tech.), anti phospho-ERK (SANTA CRUZ BIOTECH), anti ERK (Cell Signaling Tech), anti Tubulin-alpha (SIGMA ALDRICH). Membranes were then washed for 5 times with 0.1% PBST (1X PBS, 0.1% Tween® 20) and incubated with secondary Goat anti-Mouse-HRPO and Goat anti-

Rabbit-HRPO Abs (diluted at 1: 3000 for anti-mouse and 1:1000 for anti-rabbit) for 1h at RT. After extensive washing with 0.1% PBST, binding of Abs was revealed by an enhanced chemiluminescence detection system. Blots were acquired by using the instrument mini HD (Uvitec, Cambridge, UK). Each band was converted by mini HD into a densitometric trace allowing calculations of intensity and signals normalized on the signal of actin, used as the housekeeping protein, and also on the signal of total proteins, in the case of phosphorylating forms.

Statistics

For statistical comparison between samples, the Mann-Whitney U test was used for unpaired sample data and the Wilcoxon signed-rank test for paired sample data. Analyses were performed using the GraphPad Prism version 5.00 statistical software (GraphPad Software Inc., La Jolla, CA).

Results

CpG and CpG+IL-15 stimulations induce the expression of IL23R complex and IL12R complex in CLL cells

We investigated whether the expression of the IL23R complex and IL12R complex are inducible after CpG/CpG+IL-15 stimulation in CLL cells.

As shown previously, unstimulated CLL cells show a limited expression of IL23R complex[83]. In addition, similarly to what reported elsewhere[88] we observed here a substantially lack of expression of IL12R complex on unstimulated *ex vivo* CLL cells which is mainly determined by a consistently poor expression of IL12ß2 receptor chain (data not shown).

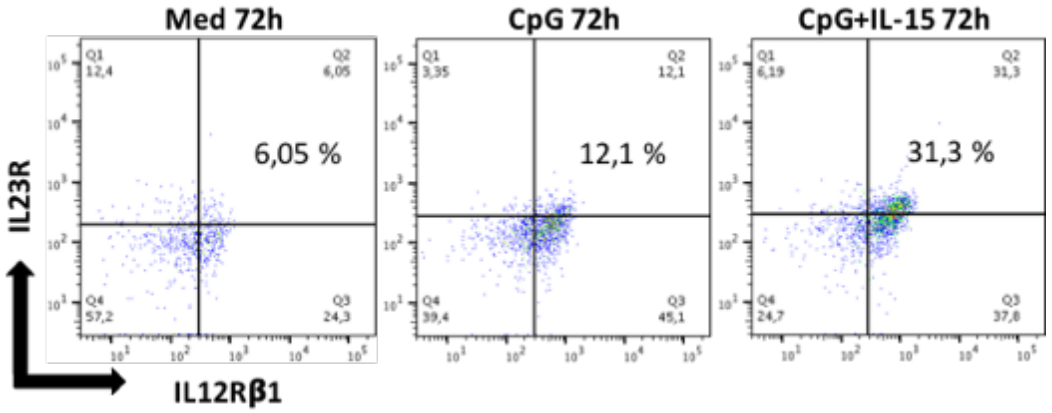
Cell activation was achieved by culturing purified CLL cells in the presence of CpG or CpG+IL15, whose synergy is important to promote CLL cells clonal expansion[70].

After 72h of stimulation a significant upregulation of surface IL23R and IL12Rß1 chains was observed whereas IL12ß2 chain was expressed on a more marginal proportion of leukemic cells. This was paralleled by a significant expression of IL23R complex and IL12R complex (Fig. 6A-B). The IL23R and IL12R complex was evaluated gating the double positive cells for both chains. Results are summarized in Fig. 6C-D.

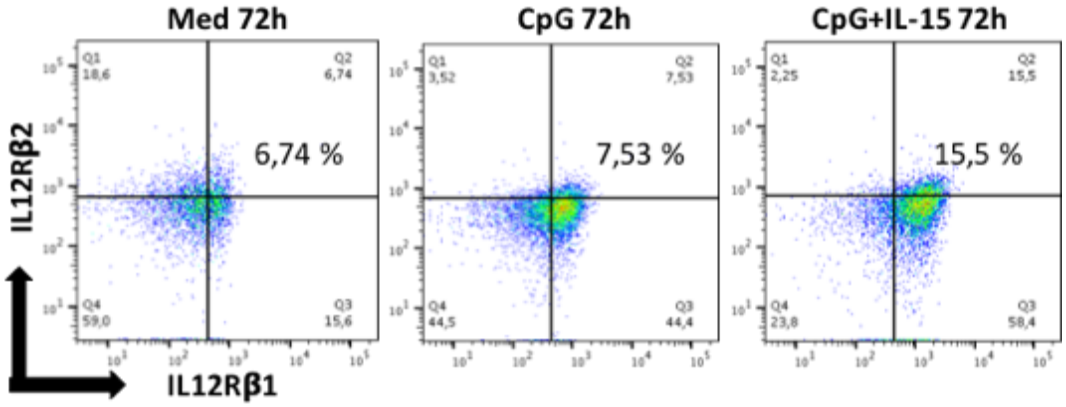
Overall, the expression of IL23R complex appears to be more sustained compared to the IL12R complex on stimulated CLL cells. This was due to the limited expression of IL12ß2 chain receptor.

Effective CLL cell activation was also evaluated by measuring the expression of CD86 (Fig. 6E).

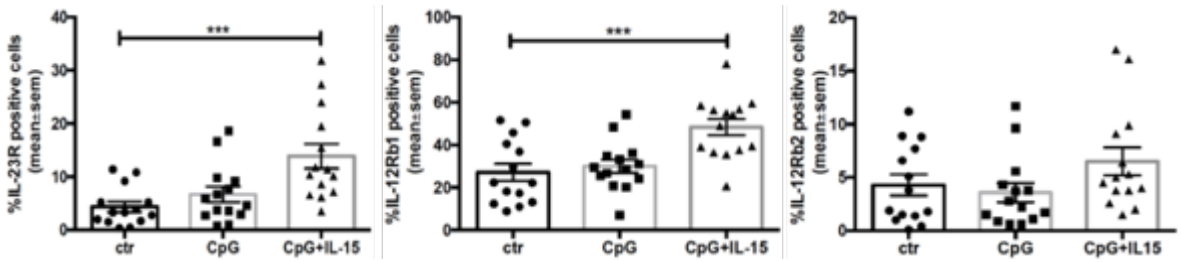
A


Med 72h
CpG 72h
CpG+IL-15 72h
6,05 %
12,1 %
31,3 %
IL23R
IL12Rβ1

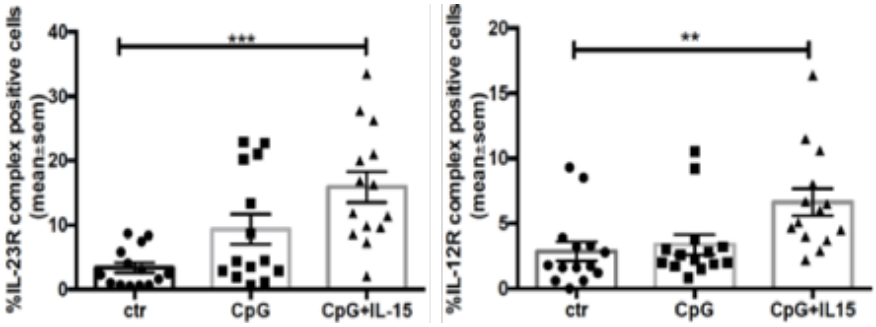
B


Med 72h
CpG 72h
CpG+IL-15 72h
6,74 %
7,53 %
15,5 %
IL12Rβ2
IL12Rβ1

C


%IL-23R positive cells (mean±sem)
%IL-12Rb1 positive cells (mean±sem)
%IL-12Rb2 positive cells (mean±sem)
***
ctr
CpG
CpG+IL-15
CpG+IL15

D



E

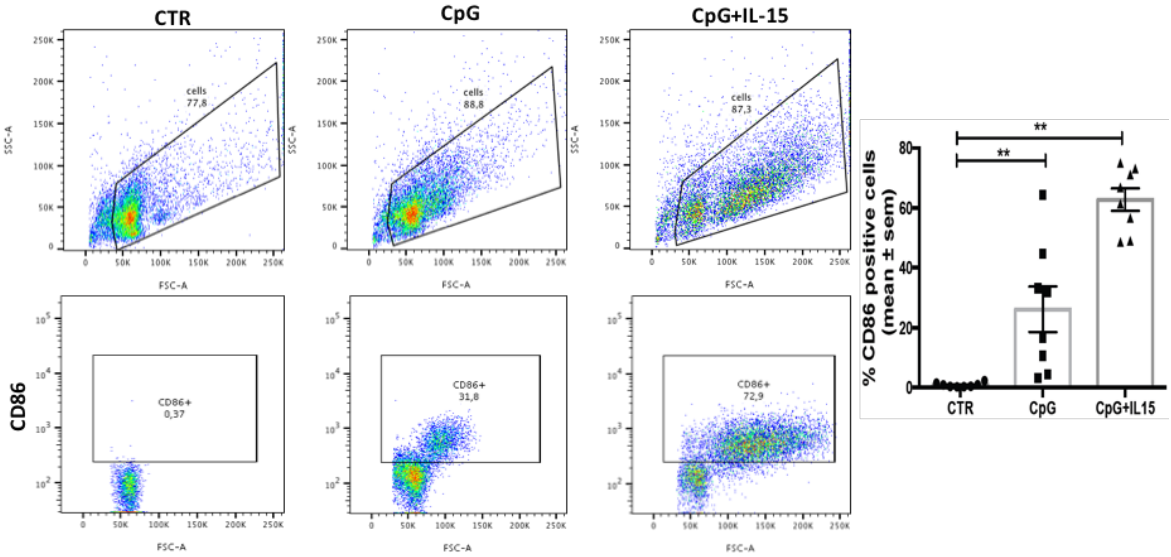


Fig. 6 IL23R and IL12R expression in CLL

A-B) Example of flow cytometry gating for the evaluation of IL23R and IL12R complex

C) Expression of IL23R, IL12Rβ1 and IL12Rβ2 chains after 72h of stimulation in 14 CLL cells samples. Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.

D) Evaluation of IL23R complex and IL12R complex expression in 14 CLL cells samples after 72h of activation. Double positive cells for both chains markers were identified within the gated viable cells. Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.

E) Example of a representative case of CD86 expression in flow cytometry after 72h of stimulation.

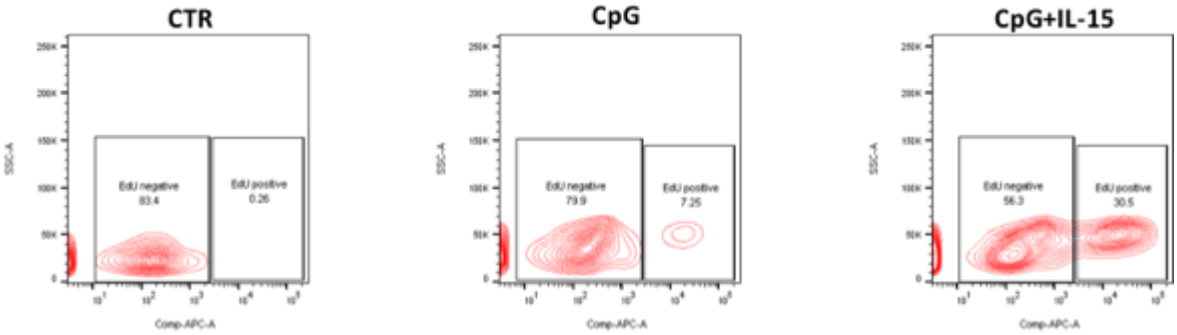
*On the right side, expression of CD86 percentage in 8 CLL cases. Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.*

Proliferating CLL cells are those that express the IL23R and IL12Rß1 receptor chains the most

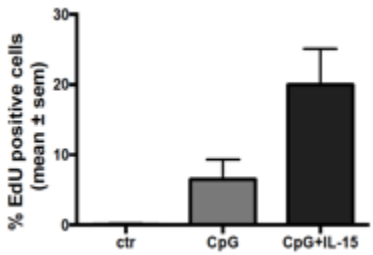
Next, we investigated whether after stimulation the cells were proliferating and whether the IL23R and IL12Rß1 receptors could be detected on proliferating cells; unfortunately we weren't able to use an antibody to detect the IL12Rß2 at the time this set of experiments was made. CLL cells were resuspended in medium with EdU and seeded in the 96 wells round bottom plate with CpG and CpG+IL15. After 4 days of culture evidence for new DNA synthesis was observed in a relevant fraction of CLL cells. The addition of IL15 was indispensable to determine higher cell division, as expected[70] (Fig. 7A-B). The upregulation of the receptor chains in the proliferating cells is presented in Fig. 7C-D.

Fig. 7E shows as, upon stimulation with CpG and CpG+IL15, the upregulation of these two receptor chains is more evident in the proliferative cells (EdU+) compared to the group of cells EdU-. However, differences did not reach statistical significance may be due the limited number of sample tested..

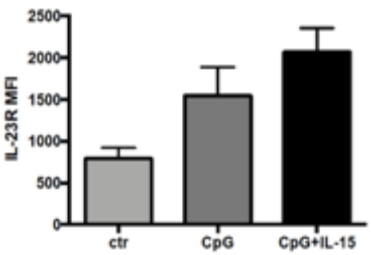
A



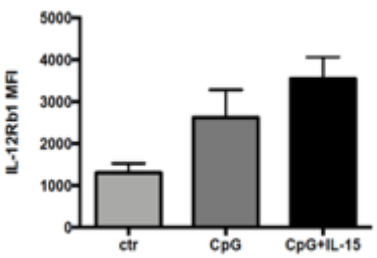
B



C



D



E

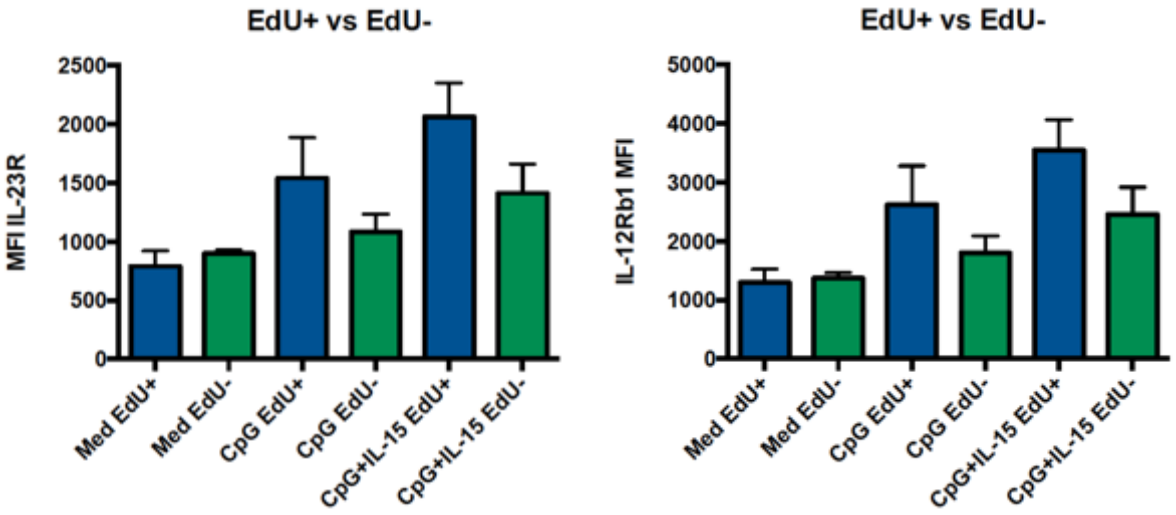


Fig. 7 Proliferating CLL cells and receptors expression

A) Example of EdU staining showing the proliferative fraction of CLL cells after 4 days of stimulation in a representative sample.
B) Representation of the percentage of EdU positive cells after 4 days of stimulation; cells were incubated with EdU 10μM, washed and stained for the receptors. After, cells were washed again and incubated with Click-IT solution for 30' RT. Before Flow analysis cells were washed and resuspended in FACS buffer (left side).
C-D) Expression of the 2 chains in MFI within EdU positive cells (right side of the figure; n=4).
E) Representation of IL23R and IL12Rβ1 chains in EdU+ and EdU- cells (MFI). Statistical difference was not reached between the groups compared.

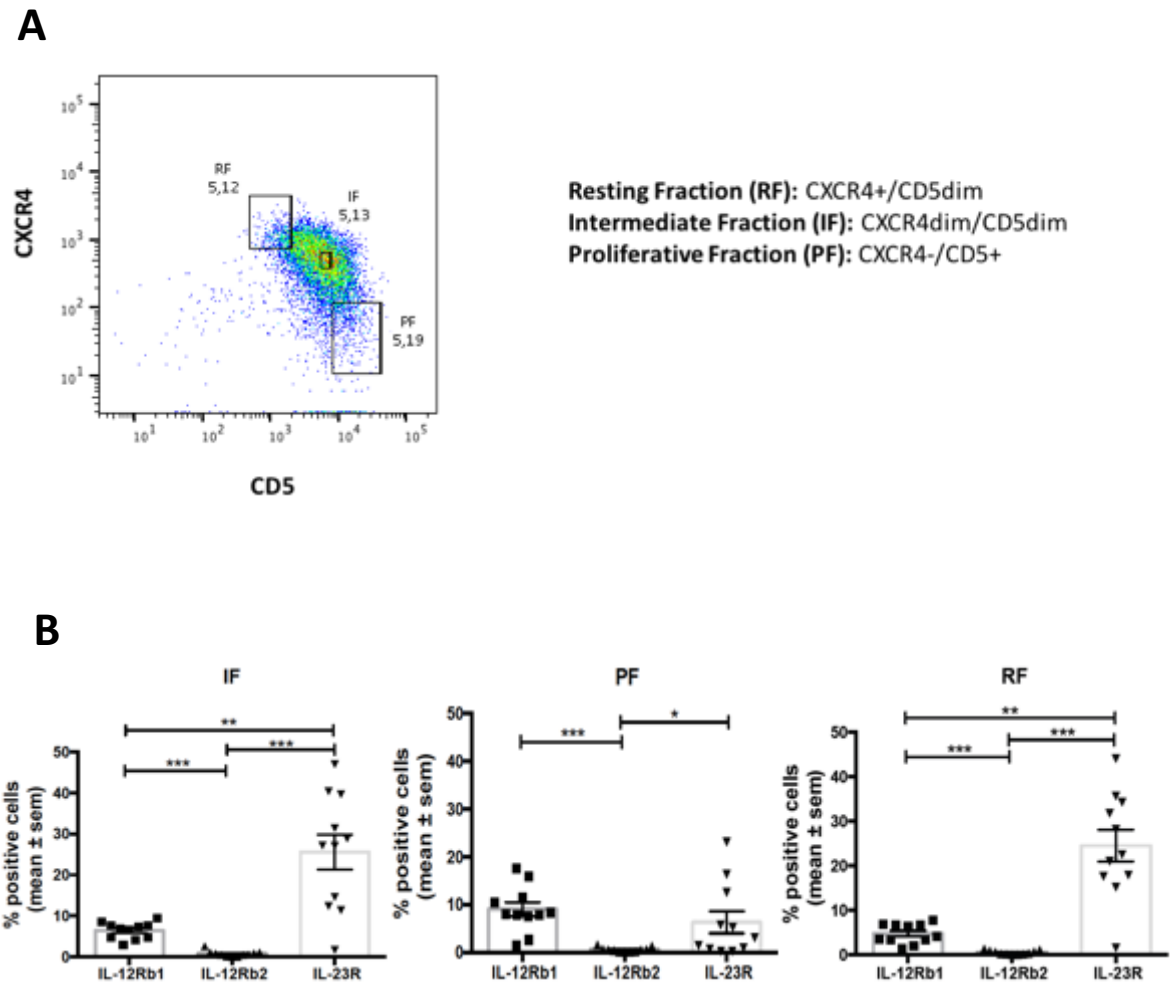
IL23R and IL12R chains expression is heterogeneous in CLL fractions

Calissano et al described for the first time a hypothetical model of a lifecycle of CLL cells[87]. During the first part of the lifecycle the B cells rest in the stroma because of the CXCR4-CXCL12 interactions. After stimulation, cells start to proliferate, upregulate the CD5 and internalize the CXCR4. These cells, with low CXCR4 expression (CXCR4dim/CD5bright phenotype), can exit the solid tissue and reach the peripheral blood. These cells have been defined as Proliferating Fraction (PF). It should be noted that these cells have to be considered as "recently divided" rather than "actively proliferating". Once these cells reach the blood, because of a lack of trophic input from the solid tissue microenvironment, they re-express CXCR4 and downmodulate CD5. This fraction has been termed as Intermediate Fraction (IF). At this point the cells CXCR4int/CD5int change their phenotype in CXCR4bright/CD5dim.

The re-expression of CXCR4 give to CLL cells the chance to reenter in the lymphoid tissue following the CXCR4/SDF1 gradient. This last fraction (CXCR4bright/CD5dim) has been

termed as Resting Fraction (RF). Fig. 8A depicts phenotypic features of CLL fractions as defined above.

Based on this separation, we wondered whether the CLL cells preferentially express the IL23/IL23R complex and the IL12R complex at certain activation/maturation stage. To do that we checked the expression of the different receptors for each CLL fraction.
We observed a similar expression of IL12Rß1in the 3 different fractions with an apparent increment in the PF, whereas expression of IL12Rß2 was substantially absent in the 3 fractions (Fig. 8B). We observed a lower percentage of cells expressing IL23R in the PF compared to the IF and RF.



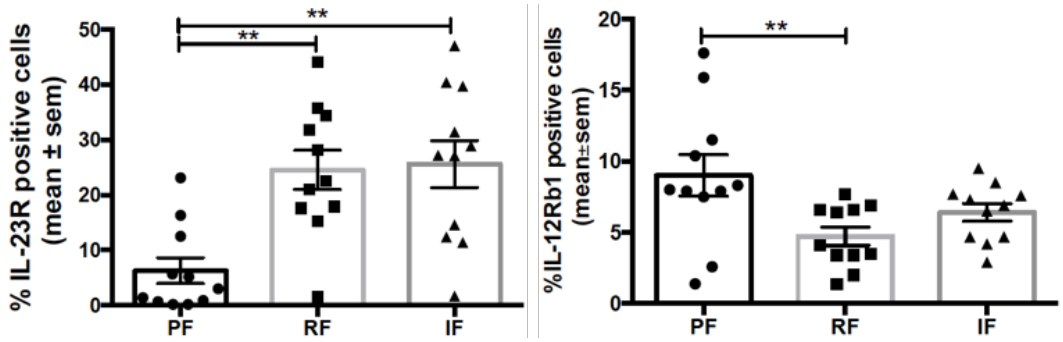


Fig. 8 CLL gating to identify CLL fractions and expression of the receptor chains in each CLL fraction.

A) Example of gating strategy of CLL fractions
B) Expression of IL23R, IL12Rβ1and IL12Rβ2 in CLL fractions in 12 CLL patients.
*Below is represented the expression of IL23R and IL12Rβ1 in each fraction. Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.*

Expression of IL23R and IL12R in normal B cells

Next, we investigated whether the expression of IL23R or IL12R can be induced in normal B cells can help solving the issue of whether the activation of the loop represents an ectopic feature characterizing CLL cells. Preliminary analysis showed that IL23R and IL12R complexes were virtually absent in total PB B cells from normal donors (Fig. 9A, C).

Total B cells were isolated and cultured in the presence of CpG and CpG+IL15. The expression of the receptors was evaluated at time 0 and at 72h for each condition. Indeed, the expression of IL23R and IL12R complexes was consistently observed in a variable proportion of stimulated B cells (Fig. 9A, C). A modest quota IL23R and IL12R complex was also observed in unstimulated B cells, maybe indicating some level of activation in the culture conditions. In addition, we evaluated whether these IL23R and IL12R complexes positive cells were represent by naïve or memory B cells as defined by the expression of IGD/CD27.

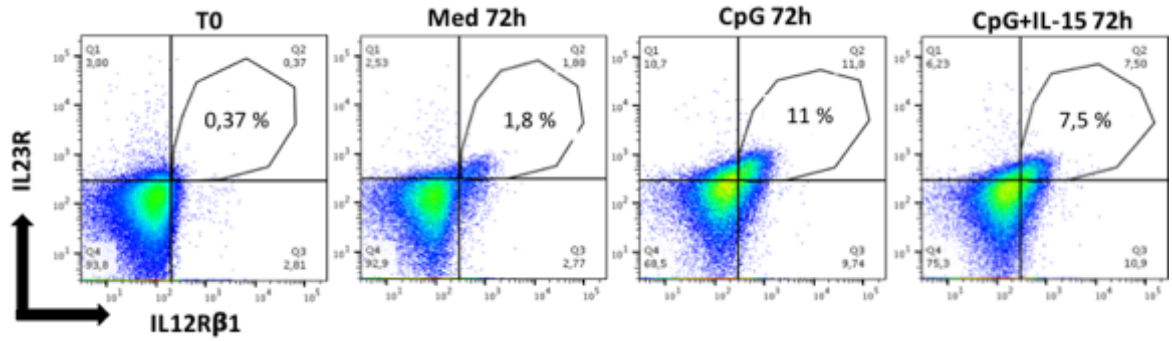
Upon stimulation with CpG/CpG+IL15, IL23R complex positive cells appeared mostly composed of CD27+ cells (memory B cells) compared to CD27- naïve B cells (Fig. 7E).

At variance, the IL12R complex positive B cells observed upon stimulation with CpG/CpG+IL-15 were similarly represented by naïve and memory B cells (Fig. 7E).

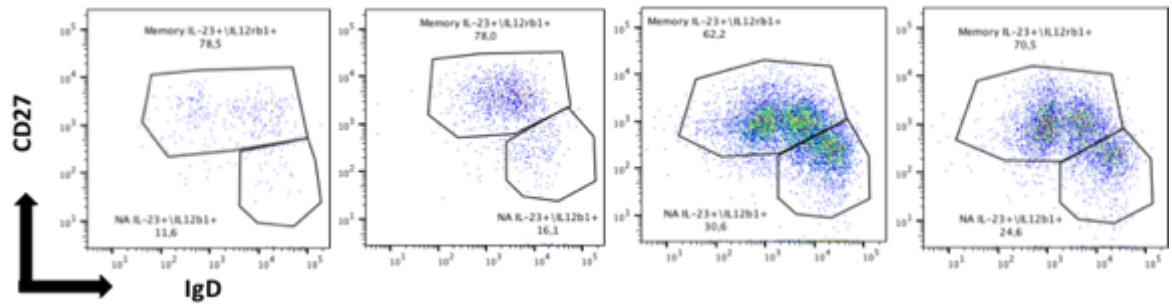
In addition, most of IL12Rß2+/IL12ß1- B cells observed after stimulation were represented by naïve B cells (Fig. 7E).

After these observations the expression of these receptors in CLL and normal B cells was compared upon stimulation CpG/CpG+IL15 (Fig. 7F). We observed that the IL23R complex is expressed in a higher proportion of CLL cells whereas the IL12R complex is expressed in a higher proportion of stimulated B cells. Similarly, the IL12Rß2 chain is represented in a higher percentage of stimulated normal B cells compared to stimulated CLL cells.

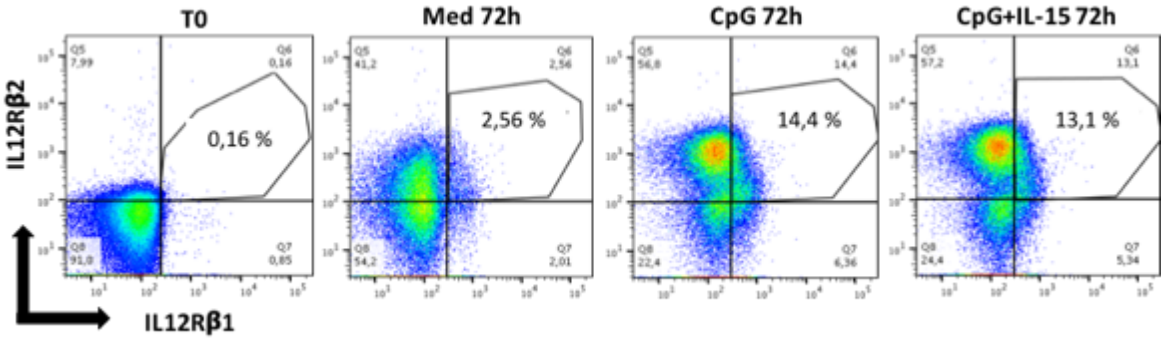
A



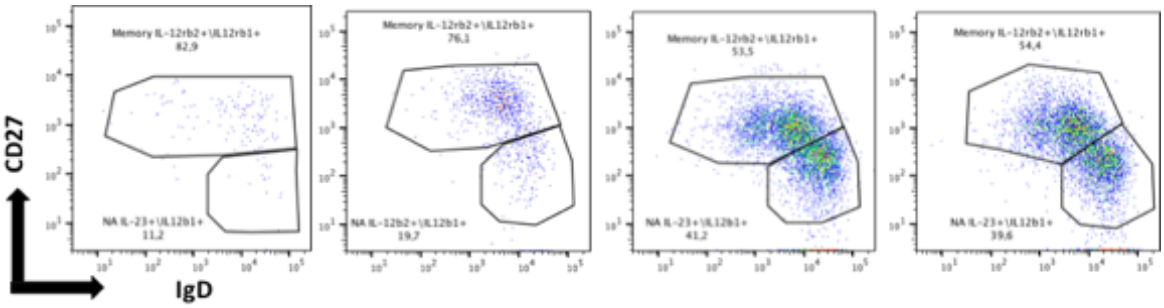
B



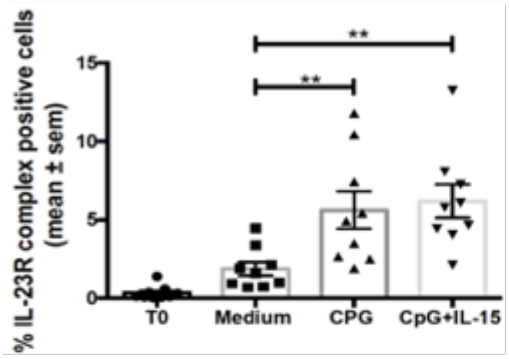
C

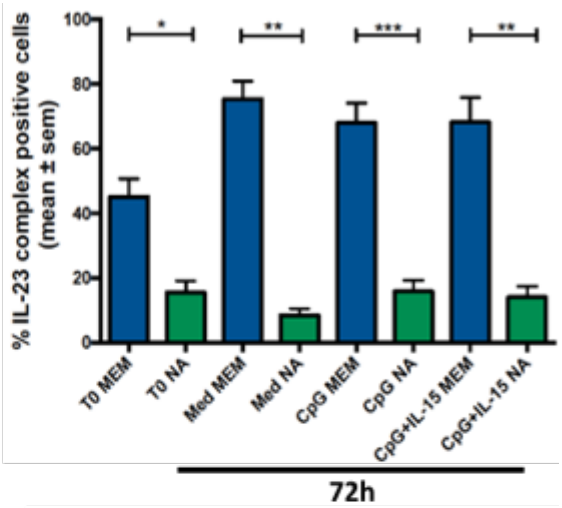

T0
Med 72h
CpG 72h
CpG+IL-15 72h
IL12Rβ2
IL12Rβ1
0,16 %
2,56 %
14,4 %
13,1 %

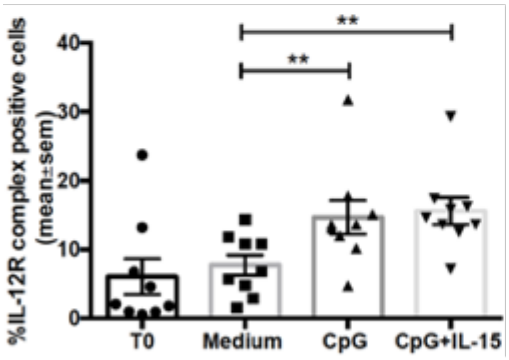
D

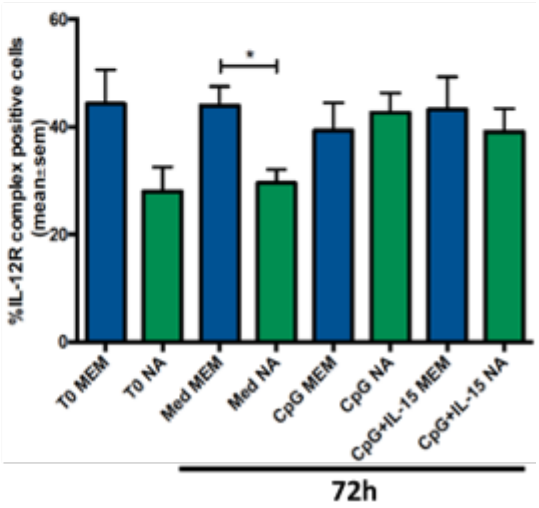

CD27
IgD

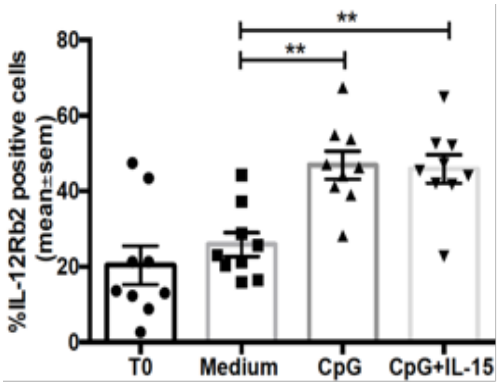
E

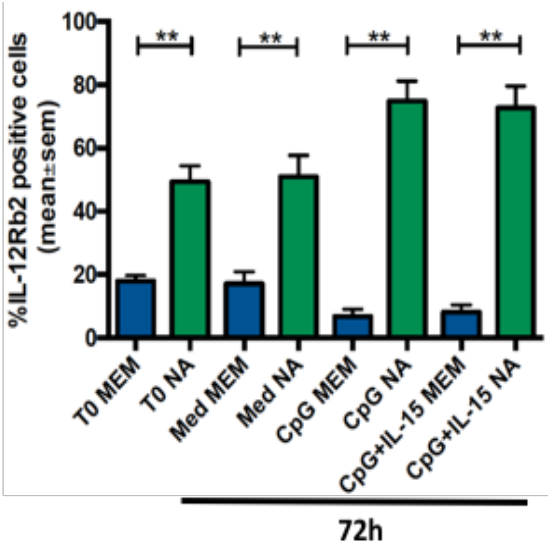

% IL-23R complex positive cells (mean ± sem)
15
10
5
0
**
**
T0
Medium
CPG
CpG+IL-15


% IL-23 complex positive cells (mean ± sem)
100
80
60
40
20
0
*
**
***
**
T0 MEM
T0 NA
Med MEM
Med NA
CpG MEM
CpG NA
CpG+IL-15 MEM
CpG+IL-15 NA
72h


%IL-12R complex positive cells (mean±sem)
40
30
20
10
0
**
**
T0
Medium
CpG
CpG+IL-15


%IL-12R complex positive cells (mean±sem)
60
40
20
0
*
T0 MEM
T0 NA
Med MEM
Med NA
CpG MEM
CpG NA
CpG+IL-15 MEM
CpG+IL-15 NA
72h


%IL-12Rb2 positive cells (mean±sem)
80
60
40
20
0
**
**
T0
Medium
CpG
CpG+IL-15


%IL-12Rb2 positive cells (mean±sem)
100
80
60
40
20
0
**
**
**
**
T0 MEM
T0 NA
Med MEM
Med NA
CpG MEM
CpG NA
CpG+IL-15 MEM
CpG+IL-15 NA
72h

F

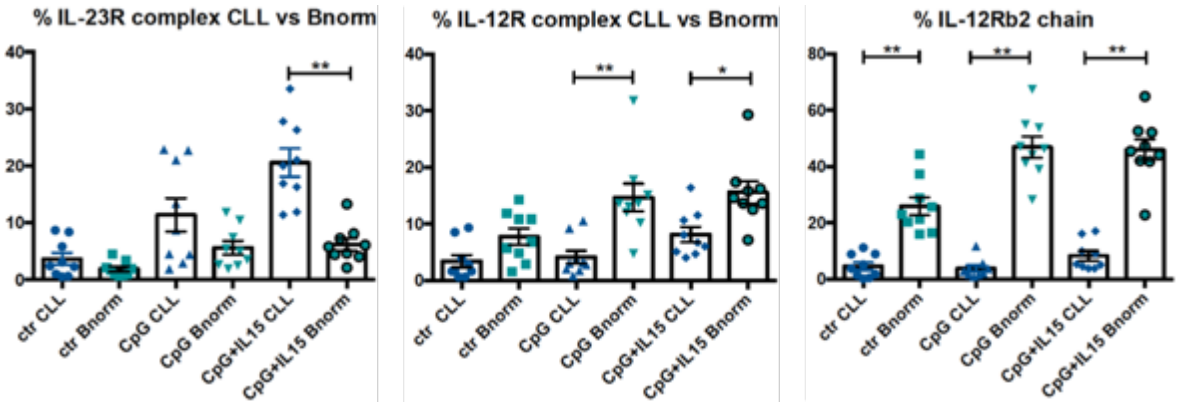


Fig. 9 Expression of IL23R complex and IL12R complex by normal B cells.
A-B) Flow cytometry profiles obtained in a representative experiment; the percentage of IL23R double positive cells at T0 and after 72h of culture as indicated (A).
The cells IL23R complex positive were further analyzed for CD27 and IgD expression to identify the Memory cells (CD27+ IgD-) and the Naïve cells (CD27- IgD+) subpopulations (B).
C-D) Flow cytometry profiles obtained in a representative experiment; the percentage of IL12R double positive cells at T0 and after 72h of culture as indicated (C).
The cells IL12R complex positive were further analyzed for CD27 and IgD expression to identify the Memory cells (CD27+ IgD-) and the Naïve cells (CD27- IgD+) subpopulations (D).
E) Receptors expression in normal B cells at time zero and after 72h of stimulation (on the left) and expression of these receptors in Memory (blue column) and Naïve (green column) subpopulations (right side of the figure). The data indicate that the receptors and IL12Rß2 chain increase their expression after stimulation. Of note, Memory B cells can express significantly higher percentages of IL23R complex positive cells than Naïve B cells upon stimulation with CpG while Naïve cells can express high levels of IL12Rß2. (n=9).
*Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; *** p 0.001; ****p 0.0001*
F) Comparison of receptors expression between CLL cells and normal B cells. Apparently IL23R complex is higher in the CLL cells while IL12R complex and IL12Rß2 are higher in the normal B cells. (n=9)
*Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; *** p 0.001; ****p 0.0001*

IL23 and IL12 secretion from CLL and normal B cells after stimulations

To better understand if activated CLL cells can not only express the receptor, but also produce the cytokines to activate an autocrine loop, we tested the supernatants with an Elisa kit.

In this set of experiments, we evaluated CpG compared to CD40L stimulation of CLL cells Based on a previous report[83] CD40L stimulation was used as a positive control to evaluate IL23 secretion[83].

As shown in Fig. 10A we observed, as expected, that the stimulation with the CD40L induces the production of IL23p19. In addition, the detection of IL12p70 was observed as well.

Overall, CpG induces a lower production of IL23p19 compared to CD40L and a limited production of IL12p70 that is, however, significant if compared to the unstimulated cells.

After this first set of experiments, the production of IL23p19 and IL12p70 of normal B cells after 72h stimulation with CpG was compared to the production of these cytokines by CLL cells in the same culture conditions (Fig. 10B).

Normal B cells showed a modest basal production of IL23p19 cytokine which increases after their stimulation similarly to what observed with CLL cells. IL12p70 was mostly observed in normal B cells upon CpG stimulation. However, compared to CLL cells, differences were not statistically significative likely due to the still limited number of samples tested.

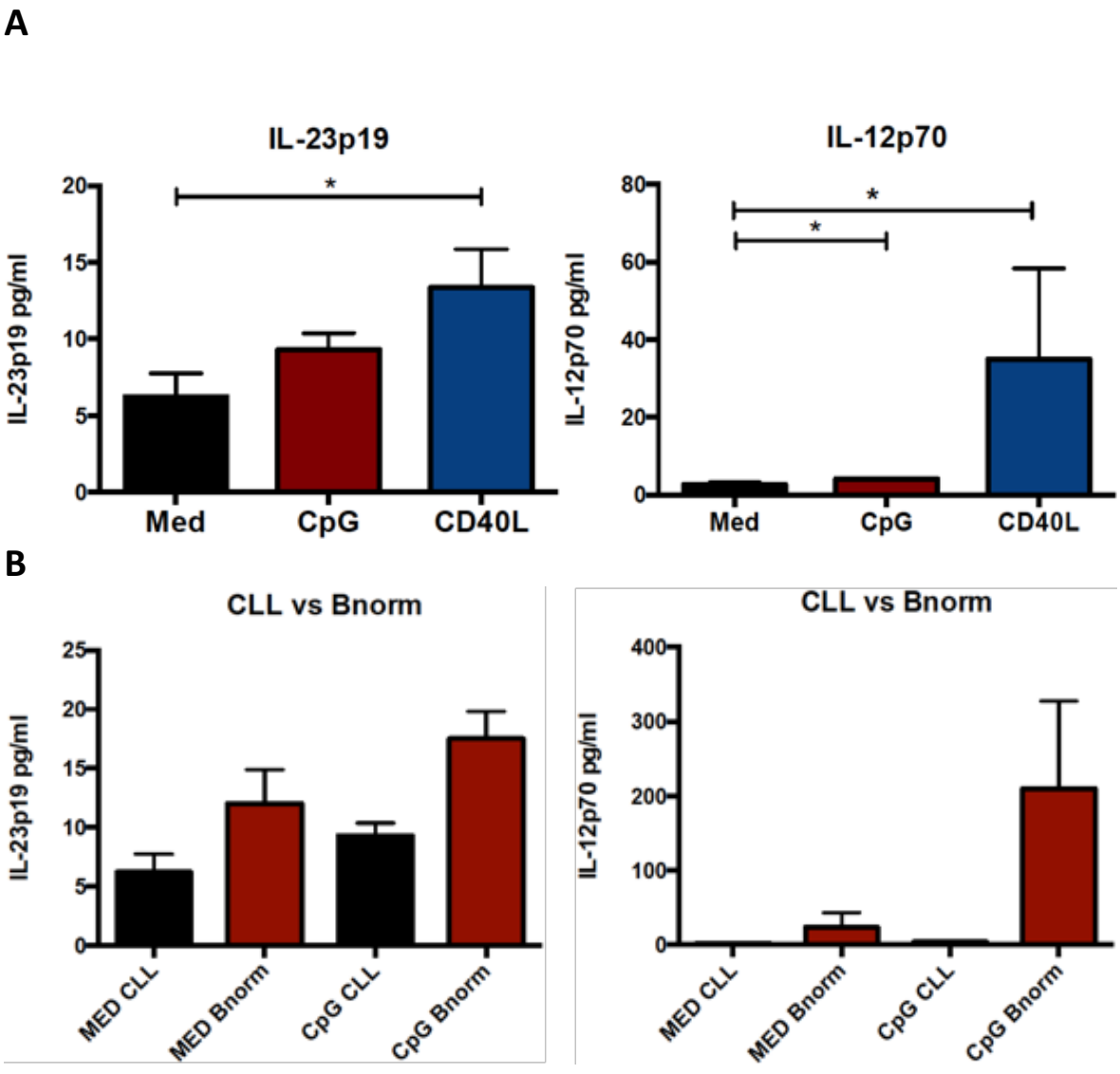


Fig. 10 Production of IL23p19 and IL12p70 in CLL and Normal B cells supernatants

A) Supernatants of CLL experiments (n=6) were collected after 72h of stimulation and tested with Luminex kit for IL23p19 and IL12p70.

*Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001*

B) Comparison of IL23p19 and IL12p70 secretion in CLL cells and normal B cells (statistical difference was not reached).

CLLs activated can signal through the JAK-STAT pathway

Finally, we investigated whether the complete receptors expressed in the membrane after stimulation are functioning receptors.

To do that, CLL cells were cultured for 48h with CpG. Cell were accurately washed and stimulated with the set of cytokines of the IL12 family for 30'. In particular we tested: IL23, IL12 and IL35. This last cytokine was included because it shares one receptor's chain (IL12Rß2) and one cytokine subunit (IL12p35).

Related to the JAK-STAT pathway, we tested the signaling for the main pSTAT involved for these receptors reported in the literature[89]: pSTAT3, pSTAT1 and pSTAT4.

Fig. 11 shows a representative experiment for WB analysis. Overall, IL12 and IL23 were capable to induce substantial phosphorylation of STAT1 and STAT3 compared to STAT4 which was mostly phosphorylated follow incubation with IL35.

In addition, an increased amount of pERK was observed following incubation with all the cytokines tested.

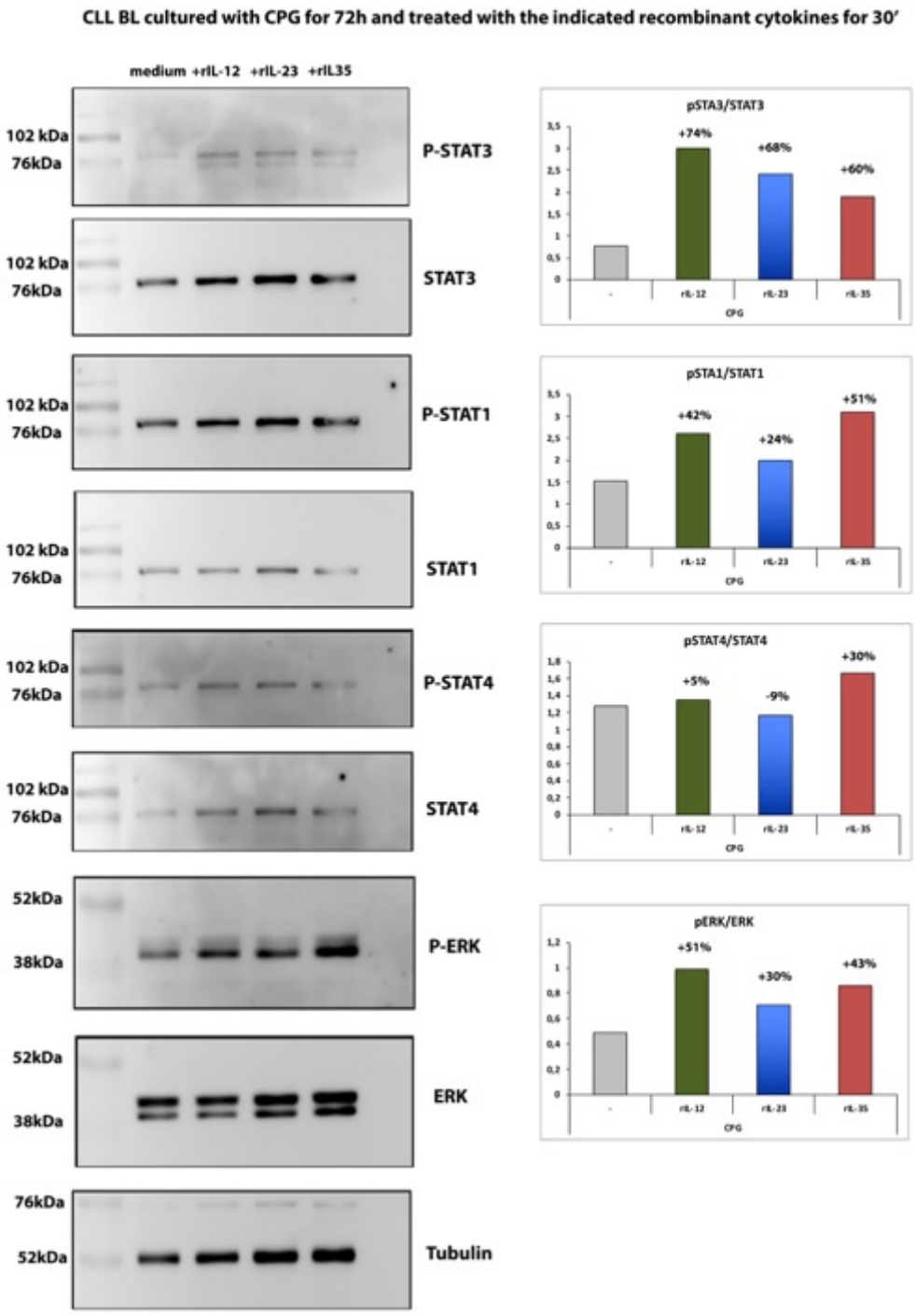


Fig. 11 *Tyrosine phosphorylation of specific proteins (as indicated) in cells from a representative CLL case stimulated with CpG for 72h and subsequently exposed to the indicated cytokines for 30'. Protein bands measured by Image-J software and ratios of the phosphorylated vs total proteins were plotted in the histograms* on the left side.

Discussion

More and more studies are focused on the importance of the micro-environment in cancer diseases. As for CLL, it is well recognized that expansion of leukemic clones requires the interaction with accessory cells and with cytokines present in the micro-environment[4,90]. These interactions likely occur primarily in the proliferating centers of peripheral lymphoid tissues, although the exact mechanisms involved are far from being elucidated. Recently, it has been demonstrated that IL23 (a cytokine of the IL12 family) plays an important function for CLL cell survival and expansion[83]. Upon interaction with activated T cells or with other CD40L-expressing cells, CLL cells express a functional IL23R complex and secrete IL23 in vitro, causing the formation of an autocrine/paracrine loop, which promotes cell proliferation[83].

In this study we explored whether IL23R/IL23 loop could be activated upon T-cell independent stimuli (such as CpG/IL15) of CLL cells. In addition, we have evaluated the possibility that another member of IL12 cytokine family (namely IL12 and its receptor) can be expressed as well on stimulated CLL cells. In addition, the expression of these receptor chains and cytokines was explored in purified B cells derived from normal donors.

Expression of IL23R/IL23 in CLL cells upon stimulation with CpG

The first evidence that we provide is that IL23R complex is expressed on CLL cells upon stimulation with CpG. The expression of the IL23R complex is more evident when IL15 is added to the cell culture (Fig. 6A). The addition of IL15 favors the cell cycle entry of leukemic cells[70] (Fig. 7). Indeed, a higher proportion of leukemic cells that have initiated DNA duplication express IL23 receptor chains (Fig. 7C-D).

As CD40L/CD40 stimulation of CLL cells has been demonstrated capable of inducing IL23 secretion, the presence of this cytokine has been tested upon CpG stimulation on culture supernatants. A lesser amount of IL23 was detected of CLL cells supernatant upon CpG stimulation compared to the stimulation obtained through CD40 molecule. This would indicate that the full IL23R/IL23 loop might not be obtained by CpG stimulation of CLL cells only. However, it can be envisaged that other sources of IL23 can be available in lymphoid tissues[91] capable of binding the IL23R complex.

Expression of IL12R/IL12 in CLL cells upon stimulation with CpG

Studies related to the expression/secretion of IL23R complex/IL23 were paralleled with those on IL12R complex/IL12. IL12 receptor complex shares the IL12ß1 chain with IL23R complex and depends on the expression of the IL12ß2 chain for its functionality. We observed that, with few exceptions, the expression of this last receptor chain is less pronounced compared to the IL23R chain (Fig. 6B-C). This is reflected in a limited proportion of leukemic cells expressing the full IL12R (Fig. 6D).

Upon stimulation of CLL cells with CpG the secretion of IL12 was rather limited as observed for IL23 (Fig. 10A). Apparently the CLL cells stimulation through CD40 was more capable to induce IL12 secretion, however this has to be confirmed in a higher number of samples.

Expression of IL23R and IL12R complexes in PB normal B cells

To better understand whether what we observed in CLL cells was comparable to B cells derived from the PB of normal donors we reproduced these stimulations on purified normal B cells.

The expression of the IL12 and IL23 receptor chains was virtually absent in unstimulated PB B cells. Upon CpG stimulation the IL23R and IL12Rß1 was identified in a limited proportion of B cells as opposed to the IL12ß2 receptor which was expressed on the majority of B lymphocytes (Fig. 9C-D). Indeed, only a proportion of B cells appeared to express the full IL12R complex (Fig. 9C-D). The capacity of normal B cells to express IL23 and IL12 receptor complexes compared to CLL cells revealed striking differences. The expression of IL23R complex was observed on a larger proportion of CLL cells compared to normal B cells, whereas the IL12R complex was expressed on a larger proportion of normal B cells compared to the leukemic clones. However, as mention above, the most striking difference observed between normal B cells and CLL cells was the substantial inability of the leukemic cells to express IL12Rß2 chain upon CpG stimulation. This observation would agree with previous reports indicating that this gene is highly methylated in CLL cells limiting its expression[88].

A side observation of this part of the study indicates that, upon stimulation of B cells with CpG, the expression of IL23R complex involve mainly memory B cells as defined by the expression of CD27 molecules (see Fig. 9D). As for the expression of IL12R complex, this seems to be distributed on both memory and naïve B cells. We consider this observation as

preliminary as the real solidity of these indications should be confirmed on further experiments that forecast CpG stimulation of sorted B cells (CD27+ vs CD27) which are programmed the near future. However, the finding that IL23R complex is preferentially expressed by memory B cells would reinforce the notion that CLL cells originate from memory B cells[92,93].

Expression of IL23 and IL12 receptor chains in CLL cell fractions

The CLL clone observed in the PB display a certain degree of heterogeneity as shown by the identification of "fractions" that identify leukemic cells in the PB at different stage on their route from and to lymphoid tissues[87]. We observed that IL23R chain is represented in higher proportion of RF and IF of leukemic cells compared to PF. In contrast, IL12ß1 seems to be more represented in the RF fraction (Fig. 8B). This observation might be interpreted as if the PF (which identifies recently divided leukemic cells just emerged from lymphoid tissues) had downmodulated IL23R chain. This mechanism might favor the release of leukemic cell from the lymphoid tissues. Unfortunately, we could not evaluate the real absence of the IL23R complex in PF leukemic cells, and in the other CLL fractions, as this needs a more sophisticated multiparametric cytometric analysis not doable with the reagents available at the moment.
The IL12Rß2 chain was consistently undetectable in CLL fractions in all the samples tested.

Ability of IL12R and IL23R complexes to signal in CLL cells stimulated with CpG

Preliminary experiments aimed at evaluating the ability of IL12R and IL23R complexes to signal when engaged with their cytokines indicate that pSTAT1 and pSTAT3 are readily induced. In addition, we show that also IL35 is capable of signaling on stimulated CLL cells. IL35R shares IL12Rß2 chain with IL12R complex (parallel studies related to this cytokine are being conducted and not shown in this report as still preliminary). However, we believe that the presence of cytokine receptor chains shared between several receptor complexes makes difficult to predict the behavior of CLL cells in a complex environment such as lymphoid tissues. Indeed, the net effect of cytokines stimulations can be dictated by competitions for receptor chains based on local concentration of cytokines and on their affinity for the proper receptors.

It has been reported that loss of signaling through IL12 receptor may favor malignant B-cell clonal expansion through several mechanisms[88,94] Our results suggest that, although a proportion of activated CLL cells can express a functional (signaling) IL12R complex, the concomitant presence of family related receptor chains might modulate the functionality of this receptor. However, functional experiments to evaluate the biological consequence of IL12R complex engagement on activated CLL cells, even in the presence of family related cytokines, have to be performed to better address and understand this issue.

Taken together, we demonstrated that CLL cells are capable to upregulate different receptors of the IL12 cytokine family upon TLR9 stimulation. The modulation of these receptors on activated CLL cells appears to differ from that observed in purified normal B cells indicating non-random conditioning in the mechanisms that regulate the expression of this cytokine family members.
Further studies related to the actions of these cytokines in the CLL micro-environment would be useful to understand which role they play in this disease and to understand whether they could represent a novel target for adjuvant therapies in this still incurable disease.

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