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The arachidonic acid–LTB4–BLT2 pathway enhances human B-CLL aggressiveness

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ABSTRACT

Deregulation of the oxidative cascade of poly-unsaturated fatty acids (PUFAs) has been associated with several cancers, including chronic lymphocytic leukemia (B-CLL). Leukotriene B4 (LTB4), a metabolite of arachidonic acid (AA), is produced by B-CLL and contributes to their survival. The aim of the present study was to analyze the activity of the oxidative cascade of PUFAs in B-CLL. Purified B cells from patients and normal B CD5 positive cells were subjected to flow cytometry, Western-blot and RT-qPCR analyses. LTB4 plasma and intracellular concentrations were determined by ELISA. Our results showed that aggressive B-CLL tumor cells, i.e. cells with an annual proliferation index above 2, over-expressed calcium-dependent and calcium-independent phospholipases A2 (cPLA2-alpha and iPLA2-beta, respectively), 5-lipoxygenase (5LOX) and leukotriene A4 hydroxylase (LTA4H). Intracellular LTB4 levels were lower in the most aggressive cells than in cells with a smaller proliferation index, despite equivalent plasma levels, and lower expression of cytochrome P450 4F3A (CYP4F3A), one major enzyme involved in LTB4 inactivation. Since BLT2, a LTB4 membrane receptor was also more often expressed on aggressive tumor cells, and since a BLT2 inhibitor significantly impaired B-CLL viability in vitro, we propose that LTB4 was efficiently trapped onto BLT2 present on aggressive tumors, thereby eliciting an autocrine response. Taken together our results demonstrate a major deregulation of the pathway leading to LTB4 synthesis and degradation in B-CLL cells, and provide a framework for understanding how these modifications promote cell survival and proliferation, especially in the most aggressive BCLL.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult blood cancer in the Western world. CLL is characterized by the accumulation of mature-looking immune-incompetent lymphocytes. B-CLL lymphocytes are the most frequent, representing 95% of the CLL cases. B-CLL manifests a variable clinical course characterized by progressive disease and short survival in approximately half of the cases, and a relatively stable disease and normal lifespan in the remaining patients. B-CLL lymphocytic tumor cells have extended survival but low proliferative potential [1]. Apoptosis of B-CLL leukemia cells is regulated by multiple signal transduction pathways [2–5]. Several studies have stressed the importance of the medullary environment for tumor B-CLL cells survival, through cellular interactions and cytokines or hormone receptors' activation [3–5].

Activation of the oxidative cascade of polyunsaturated fatty acids (PUFAs) has been associated with several cancers, including B-CLL [6,7]. Specifically, phospholipase A2 (PLA2) activation results in arachidonic acid release that can be metabolized either into leukotrienes by lipoxygenases (LOX), or into prostaglandins by cyclooxygenases (COX) [6–9]. Cyclooxygenase 2 (COX2) expression in B-CLL tumor cells has been reported and associated with enhanced B-CLL lymphocytic tumor cells survival due to increased production of PGE2 and PGF2 [10,11]. A paracrine loop has now clearly been demonstrated, which involves non-malignant leukocytes and nurse-like cells, but contribution of an autocrine loop to B-CLL lymphocytic tumor cells survival cannot be ruled out [3,12]. B-CLL tumor lymphocytes have indeed been shown to produce leukotriene B4 (LTB4) and to express its high affinity receptor BLT1 [13]. LTB4 seems to contribute to CD40-dependent activation of chronic B lymphocytic leukemia cells [13]. CD40 engagement severely reduces B lymphocytes' apoptosis, and contributes to B-CLL cells survival [14].

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[2] This study is dedicated to the memory of Dr JP Salaün.

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Nevertheless, in addition to being secreted and to bind its receptors BLT1 and BLT2, LTB4 can also be omega-hydroxylated by CYP4F3, a member of the cytochrome P450 superfamily. This results in its inactivation and in the formation of 20-OH-LTB4, described as an inhibitor of BLT1 expression [15]. CYP4F3 expression results from the assembly of two distinct mRNAs, produced by tissue-specific alternative splicing, and encoding the CYP4F3A and CYP4F3B proteins [16]. CYP4F3A is mostly expressed in leukocytes and CYP4F3B in the liver [17]. CYP4F3 is also expressed in activated CD38 positive lymphocytes, but neither the sub-cell populations nor the isoforms have so far been characterized [17,18]. LTB4 was recently reported to control human B lymphopoiesis and stem cell differentiation and proliferation, through the activation of its receptors BLT1 and BLT2 [19,20]. LTB4 and prostaglandins also activate members of the Peroxisome Proliferator Activated Receptor (PPAR) family of transcription factors. LTB4 binds PPAR-alpha, which in turn regulates the oxidative degradation of PUFAs and their derivatives, including LTB4 [21,22]. Moreover, PPAR-gamma, another member of the PPAR family, contributes to the regulation of inflammation [23,24]. It can also bind oxidized lipids, including 15-deoxy-delta 12-14-PGJ2, a metabolite of PGD2 [23,24].

Recent studies have shown that PPAR-gamma is a key regulator of B cell lineage differentiation and proliferation. Its activation prevents interleukin-6-dependent proliferation of myeloma cells [25] and results in apoptosis of normal B murine lymphocytes, in caspase-3- and caspase-9-dependent manner [14]. Furthermore, in cooperation with other transcription factors, PPAR-gamma represses the expression of several membrane receptors, including Toll receptors involved in B-CLL leukemia cells survival [26]. The G0/G1 switch gene 2 is a PPAR-gamma target and, possibly, a PPAR-alpha target [27], while PPAR-gamma also regulates the transcription of cyclin D1 [28].

In light of these data, it seemed of interest to us to explore whether a deregulation in the activation of the oxidative cascade of PUFAs may occur in B-CLL lymphocytic leukemia cells. In the present study, we analyzed the expression of several genes and proteins involved in the arachidonic acid-dependent transduction pathway, both in low- and in high-progressing B-CLL, in comparison with normal B CD5 positive cells.

2. Material and methods

2.1. Patients

Fifty-six patients fulfilling the criteria for the diagnosis of B-CLL were enrolled in the study, and scored clinically according to Matutes et al. and Binet classification (Table 1) [29,30]. Disease stages were distributed as follows: 40 stage A, 23 men plus 17 women (50 to 90 years old, mean age 70); 11 stage B, 7 men plus 4 women (45 to 80 years old, mean age 61); and 5 stage C, 2 men plus 3 women (54 to 76 years old, mean age 68). The median follow-up was 5 years. Patients were either untreated or had not received treatment for 6 months before the study. Tonsils from 2 to 5 years old children undergoing routine tonsillectomy were also taken. Peripheral blood samples and tonsils were obtained after informed consent. The protocol was approved by the Institutional Review Board at Brest University under the reference 2008-214. Disease was considered as highly progressing when the number of B-CD5 positive lymphocytes at year n + 1 was at least twice as high as the number of B-CD5 positive lymphocytes at year n, and slowly progressing otherwise.

2.2. Cell preparation and flow cytometry

Mononuclear cells were isolated by Ficoll density-gradient centrifugation. B cells were purified by negative selection using magnetic beads (B cell isolation kit, Miltenyi Biotech) according to the manufacturer's instructions. Following isolation, more than 95% of the isolated B-CLL cells expressed CD5 and CD19, as ascertained using PE-conjugated anti-CD19, FITC anti-CD3 and PC5 anti-CD5 monoclonal antibodies and the Epics-XL FACS cytometer (Beckman-Coulter for antibodies and cytometer). For healthy B-CD5 positive cells purification from tonsils, B cells were submitted to a magnetic positive selection before cytometry analysis. The analysis of BLT1 and BLT2 membrane expression was performed on at least 5×10^5 purified cells. Antibodies were from Sigma-Aldrich, catalog numbers MFCD04118542 for BLT1 and MFCD04118543 for BLT2.

2.3. Western blotting

Total proteins were isolated immediately after cell purification, as previously described [31]. Briefly, cells (1×10^7) were lysed for 30 min on ice in a buffer containing 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 2 mM Na_3VO_4, 100 mM NaF, 4 mM NaH_2PO_4, 1 μM phenylmethylsulfoxide fluoride, 2% Triton, 2% Nonidet P40, 5 mg/ml leupeptin, and 5 mg/ml aprotinin. Equal amounts of proteins (50 μg) were loaded on 8–12% SDS polyacrylamide gels, which were then transferred onto PVDF membrane (Hybond P, Amersham), probed with appropriate antibodies and revealed by chemiluminescence. The signal was quantified using a densitometry software (Bioprofil, Vilber-Lourmat, France). Protein expression was normalized using beta-actin as an internal standard. Primary antibodies were purchased from Abcam for anti-actin (catalog number ab1801, rabbit polyclonal), iPLA2 (ab23706, rabbit polyclonal, does not cross-react with cPLA2 or sPLA2), pan-PLA2 (ab9014, sheep polyclonal), COX1 (ab695, mouse monoclonal), and COX2 (ab15191, rabbit polyclonal), from cell signaling technology for cPLA2 (catalog number 2832, rabbit polyclonal, does not cross-react with iPLA2 or sPLA2). Primary antibodies were diluted at 1/1000, except for anti-pan-PLA2 which is at 1/2500. The following reagents were purchased from GE Healthcare (Amersham Biosciences): Goat anti-rabbit IgG HRP-linked (catalog number RPN 4301, used 1/5000), sheep anti-mouse IgG HRP-linked (RPN4201, used 1/5000) and chemiluminescence kit (ECL plus, RPN 2132). Rabbit anti-sheep IgG HRP-linked was obtained from Abcam and used at 1/10,000 (ab6747).

2.4. LTB4 quantification

LTB4 was quantified from either plasma (n = 15 controls, n = 25 patients) or cell lysates (n = 5 controls, n = 25 patients). Ten million cells were lysed as recommended by the manufacturer (Cayman Biochemical) and subjected to analysis.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted with the TRIzol® (Life Technologies) solution according to the manufacturer's instructions. Total RNA (200 ng) was used for first-strand cDNA synthesis with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative RT-PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) for all transcripts except PPAR-alpha and PPAR-gamma, analyzed with specific TaqMan probes, according to the manufacturer's instructions with an ABI 7000 or 7300 real-time PCR system (Applied Biosystems). All determinations were performed in duplicate and normalized against GAPDH as an internal control gene. The results were expressed as the relative gene expression using the DeltaDeltaCt method. Fold change = 2−[(Ct target gene in sample − Ct GAPDH in sample) − (Ct target gene in normal CD5 positive B cells − Ct GAPDH in normal CD5 positive B cells)] [32]. Primer sequences are listed in Table 2.

2.6. Cell proliferation assay and reagents

Cell viability and proliferation were assessed using a MTT cell proliferation assay kit, using the optical density as a measure (570 nm) as recommended by the manufacturer (Cayman Chemical). Purified B

Table 1
Patients' characteristics (proliferation index was calculated by dividing the number of B-CD5 positive lymphocytes at year n + 1 by the number of B-CD5 positive lymphocytes at year n).

Patient number	Age (years)	Gender	Lymphocytes (10^9/L)	Binet stage	%CD38 pos cells	Proliferation index	Matutes score	Follow-up (years)
1	74	M	45	A	3	1.01	5	7
2	54	M	48	A	1	1.20	4	9
3	68	M	15	A	4	1.02	5	2
4	75	M	35	A	2	4.36	4	4
8	77	F	31.7	A	72	3.20	5	10
9	58	M	21.2	A	0	1.43	3	5
12	72	M	22	A	0	1.10	4	11
13	50	M	24	A	2	1.30	4	2
14	67	M	23	A	0	1.20	5	3
16	78	F	33	A	7	4.60	5	8
17	83	F	50	A	0	1.10	5	4
20	55	M	35	A	0	1.03	5	5
22	76	M	21.4	A	2	3.20	4	3
24	58	F	25	A	1	1.28	3	9
25	76	F	40.7	A	8	2.82	4	4
26	78	F	40	A	3	1.32	4	10
27	60	F	47.8	A	0	1.27	5	1
28	59	M	19	A	5	2.84	5	3
29	56	M	26	A	1	1.19	4	2
30	76	F	22	A	0	1.00	4	7
31	76	F	20	A	6	1.60	3	3
32	79	F	25	A	0	1.16	3	10
35	81	M	27.5	A	2	1.14	5	1
36	80	M	25	A	0	1.76	4	2
37	73	F	25	A	16	1.19	5	8
39	61	M	18	A	9	1.06	5	1
40	62	M	44	A	0	1.75	5	7
42	75	M	64	A	0	3.62	5	4
43	82	M	74	A	0	1.89	4	8
44	90	M	55	A	1	1.13	4	5
45	73	F	52	A	2	2.05	3	7
46	71	M	30	A	1	1.03	4	1
48	70	M	33	A	3	1.48	3	5
49	62	F	17	A	1	1.06	3	3
50	67	F	29	A	2	1.21	3	5
53	77	F	21	A	0	2.38	5	11
54	81	F	85.1	A	1	1.28	5	7
55	72	M	46	A	6	1.24	4	5
18	58	M	25	A	1	1.30	4	5
57	73	F	32	A	13	4.63	5	1
10	45	F	68	B	1	2.85	4	1
11	50	M	22	B	2	1.60	5	3
15	58	F	37	B	37	3.20	4	5
21	74	F	57	B	3	1.21	5	2
23	80	M	59	B	7	1.12	5	2
33	77	M	50.5	B	0	1.08	4	1
41	60	F	82	B	0	1.34	5	1
47	58	M	14	B	27	1.07	5	6
51	53	M	42	B	49	2.98	4	2
52	54	M	33	B	44	2.65	3	4
56	66	M	19	B	70	10.60	2	1
5	65	F	20	C	18	10.00	5	4
6	54	M	123	C	3	6.10	5	3
7	76	F	110	C	54	7.85	3	6
34	69	F	18	C	2	8.44	4	2
38	57	M	120	C	31	8.50	4	2

cells were incubated in duplicate for 36 h in a 96 wells plate at a density of 5×10^4 cells per well in RPMI 1640 medium with 10% fetal calf serum. To address whether 12-HHT, a metabolite of PGH2 through the action of thromboxane synthase (TXAS), may be involved in B-CLL cells survival, 20 μM ozagrel, a synthetic TXAS inhibitor, was added to the medium. The U78502 anti-BLT1 specific and LY255283 anti-BLT2 specific inhibitors were also used at 1.5 μM and 10 μM, respectively. All the inhibitors were added 30 min before adding LTB4 (150 nM).

2.7. Statistical analysis

Comparisons between groups were performed using Chi-square test or ANOVA1 test and Bonferroni correction with the PRISM Software. For quantification, expression is given as mean ± SEM.

3. Results

3.1. Expression of c- and i-PLA2

To determine whether B-CLL cells and their normal counterparts, normal B CD5 positive lymphocytes, expressed PLA2 isoforms, we made use of a pan-PLA2 antibody in a Western-blotting analysis. Two major bands of 85 and 77 kDa were distinguished, that may respectively correspond to cPLA2-alpha and iPLA2-beta (data not shown). To confirm this result, we used antibodies specific for either cPLA2 or iPLA2, with actin as a loading control. As shown in Fig. 1A, both proteins, cPLA2-alpha and iPLA2-beta, could be detected in all specimens (n = 12 controls and n = 56 patients). No truncated forms of iPLA2 were detected in any of the specimens analyzed. The expression of iPLA2-beta

Table 2
Primers' list.

Expression analyzed	Primers' sequences
GAPDH	Forward: 5′GAGTCAACGGATTTGGTCGT3′ Reverse: 5′TTGATTTTGGAGGGATCTCG3′
5LOX	Forward: 5′ACTGGAAACACGGCAAAAAC3′ Reverse: 5′TTTCTCAAAGTCGGCGAAGT3′
LTA4H	Forward: 5′ACCCTGCTACCTGATTGCTT3′ Reverse: 5′TTTCTCAAAGTCGGCGAAGT3′
TXAS	Forward 5′GGTTTTTGGGAAAGCCAAAT3′ Reverse: 5′GGACTGAAAGCAGACATCAGG3′
CYP4F3A	Forward: 5′ATTGGTTCTTGGGTCACCTG3′ Reverse: 5′CCACCAGCAGCACATATCAC3′
PPAR-alpha	Ref. Hs 00231882_m1(FAM) Sequences are the property of Applied Biosystems
PPAR-beta	Ref. Hs 00234592_m1(FAM) Sequences are the property of Applied Biosystems

was increased in leukemic cells compared to normal cells (Fig. 1B). However, no difference was found according to disease stage. Although cPLA2-alpha expression was not higher in stage A leukemic cells ($n = 40$) than in normal B CD5 positive cells ($n = 12$), an increase was observed for B and C stages ($n = 11$ and $n = 5$, respectively) in comparison with normal B CD5 positive cells and stage A leukemic cells. The same analyses were then performed to see whether variations could be observed for a same patient (Fig. 2). Tumor cells were collected every six months for $n = 43$ patients over the follow-up time. Twenty-four patients with a slowly progressing disease were analyzed (18 stage A and 6 stage B patients) and 19 patients with a rapidly progressing disease (9 stage A, 5 stage B and 5 stage C patients). In the group with a slowly progressing disease (less than one cell population doubling per year), none of the PLA2 isoforms showed a significant variation of expression within 36 months of follow-up time (Fig. 2A). In contrast, iPLA2-beta and cPLA2-alpha increased over a 2 year period for the patients with a rapidly progressing disease. This was specially observed for cPLA2-alpha, whose expression level strongly correlated with this parameter ($p < 5 \times 10^{-3}$), as compared to iPLA2-beta ($p = 0.01$) (Fig. 2B).

3.2. Expression of COX, 5LOX, LTA4H, and TXAS

As PLA2 activation results in the generation of bioactive lipids, and especially arachidonic acid that enters either the lipoxygenases or the cyclooxygenases pathways, the expression of cyclooxygenases 1 and 2 (COX1, COX2), thromboxane synthase (TXAS), 5-lipoxygenase (5LOX) and leukotriene A4 hydrolase (LTA4H), was further analyzed. GAPDH was chosen as an invariant RNA control ($Ct = 21.64 \pm 0.35$ cycles for control cells ($n = 12$) and 22.37 ± 0.32 ($n = 45$) for purified B-CLL cells).

Five-LOX mRNA expression level was not correlated with disease stage ($n = 33$, data not shown), but it was very significantly higher in leukemic cells than in normal B CD5 positive cells (Fig. 3A, $p = 0.54 \times 10^{-9}$). Interestingly, 5LOX mRNA level also correlated with the clinical evolution, the highest fold changes being found for the rapidly progressing patients ($n = 12$, $p = 0.0016$, Fig. 3B). In parallel, LTA4H expression was very significantly raised in B-CLL cells compared to healthy B CD5 positive cells ($p = 1.26 \times 10^{-10}$, Fig. 3C). However, no statistical correlation was found with the stage of the disease. By contrast, as observed for 5LOX, the highest fold changes were those of patients with a highly progressing disease ($p = 0.0024$, Fig. 3D).

In addition, COX1 and COX2 protein levels were analyzed (with the apparent MW of 69 and 67 kDa, respectively) ($n = 53$ samples, Fig. 4A). COX1 expression remained unchanged in tumor cells compared with normal B CD5 positive cells. No correlation was evidenced with any clinical parameter (data not shown). By contrast, COX2 was slightly overexpressed in tumor cells, at similar levels according to the stage ($p = 0.003$, Fig. 4A and B). TXAS was also more expressed in B-CLL tumor cells than in healthy B CD5 positive cells ($p = 0.016$, Fig. 4C). However, no correlation was found with the stage of the disease or the clinical evolution (data not shown).

As shown in Fig. 5, COX2 expression level was also associated with the clinical outcome ($p = 0.0021$), with the highest expression observed for the patients with adverse evolution. Nevertheless, this was not observed for TXAS ($p = 0.70$, $n = 22$ patients, data not shown).

3.3. Expression of CYP4F3

A consequence of 5LOX and LTA4H activity is the possible production of LTB4 that can further either be inactivated by CYP4F3, or trapped onto its receptors BLT1 and BLT2, thereby eliciting a biological response. RT-qPCR analyses with specific primers demonstrated that only CYP4F3A, but not CYP4F3B, was expressed in normal B CD5 positive cells and B-CLL tumor cells, in agreement with previous reports [15–17]. CYP4F3A was more expressed in B-CLL tumor cells than in healthy CD5 positive lymphocytes, ranging between 29 and 2000 folds, for highly ($n = 14$) and slowly ($n = 25$) progressing cases, respectively. In addition, a strong correlation was observed between CYP4F3A over-expression and the clinical outcome, with the lowest over-expression being observed for the fastest growing tumor cells ($p = 0.0005$, Fig. 6A). The pathways that determine the fate of LTB4 include 5LOX- and LTA4H-dependent LTB4 synthesis and CYP4F3-dependent hydroxylation. Considering the balance between both synthesis and inactivation of LTB4, we calculated the ratios between the fold-changes of 5LOX and LTA4H and that of CYP4F3A. As shown in Fig. 6B and C, and in agreement with the results observed for 5LOX, LTA4H and CYP4F3A expression, this ratio strongly increased in highly proliferating tumor cells together with 5LOX ($p = 0.006$, $n = 19$ slowly progressing vs 10 highly progressing

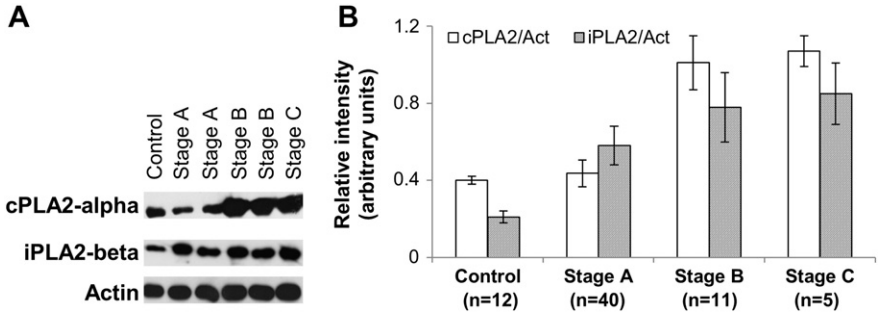

Fig. 1. Analysis of cPLA2-alpha and iPLA2-beta expression according to disease stage. A: A representative blot from a Western blotting analysis is shown. B: Mean expression normalized to actin for $n = 12$ controls and $n = 56$ patients, as determined by densitometry analysis. Control stands for healthy B CD5 positive cells (>95% purity as ascertained by flow cytometry).

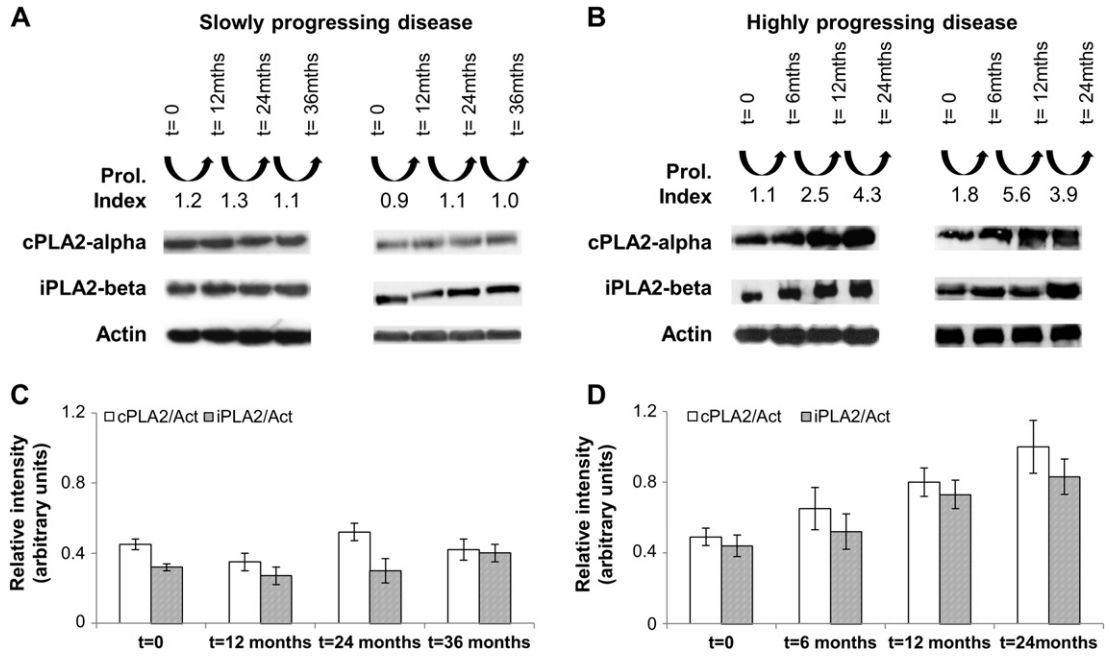

Fig. 2. Analysis of cPLA2-alpha and iPL2-beta expression and disease progression. Analysis over a 36 months follow-up for slowly progressing (A, n = 24) or 24 months follow-up for rapidly progressing (B, n = 19) disease. Two representative series of analyses are shown per group of patients. C and D: Mean expression normalized to actin (densitometry analysis) for patients with a slowly progressing disease (C, n = 24) or a highly progressing disease (D, n = 19).

cases) and LTA4H (p = 0.005, n = 15 slowly progressing vs 7 slowly progressing cases).

3.4. Expression of LTB4 receptors and LTB4 quantification

BLT1 was slightly more expressed on B-CLL tumor cells than on their normal counterparts (p = 0.045), but no correlation was found between its expression and any clinical parameter (data not shown). However, BLT2 was more often expressed on tumor cells when the disease evolved rapidly (35 to 75% positive cells, n = 10, p = 0.003) than when it evolved slowly (n = 15, Fig. 7A). In the slowly progressing condition, BLT2 expression varied from 10 to 30% of the cells analyzed and was not statistically different from that of control cells (n = 12, 10 to 20% of the cells).

LTB4 levels were analyzed independently in plasma and in cells. Plasma levels were found similar, although a slight, non-statistically significant increase was observed for patients compared to controls (1055.9 ± 114 pg/ml for n = 15 controls, and 1512.9 ± 415.4 pg/ml for n = 25 patients, p > 0.05). No correlation was found with any clinical parameters. However, concentrations in lysates of patient cells (220.26 ± 16.86 pg LTB4/μg of proteins, n = 25) were significantly higher than those of control cells (121.03 ± 11.23 pg/μg, n = 10, p = 0.0087). A correlation was found (p = 0.0023) between LTB4 intracellular concentration and the stage of the disease when values for stage A patients (253.25 ± 19.25 pg/μg, n = 17) were compared with those obtained for stage B or C patients (150.15 ± 14.62 pg/μg, n = 8). Moreover, low intracellular concentrations also correlated with an adverse evolution (p = 0.0008) with concentrations of 262.33 ± 19.25 pg/μg for patients with a slowly evolving disease (n = 15) and 157.14 ± 14.29 pg/μg for those with a rapidly evolving disease (n = 10, Fig. 7B). In the last case, concentrations were closed to those observed in normal B CD5 positive lymphocytes (121.03 ± 11.23 pg/μg, p = 0.1006, Fig. 7B).

3.5. PPAR expression

As LTB4 and prostaglandins bind and activate PPARs, which, in turn regulate cellular proliferation and differentiation, PPARs' expression was determined using RT-qPCR. Both PPAR-alpha and PPAR-gamma were over-expressed in leukemic cells in comparison with healthy B CD5 positive cells. A major increase was observed for PPAR-alpha (around 200-fold), while fold changes for PPAR-gamma ranged from

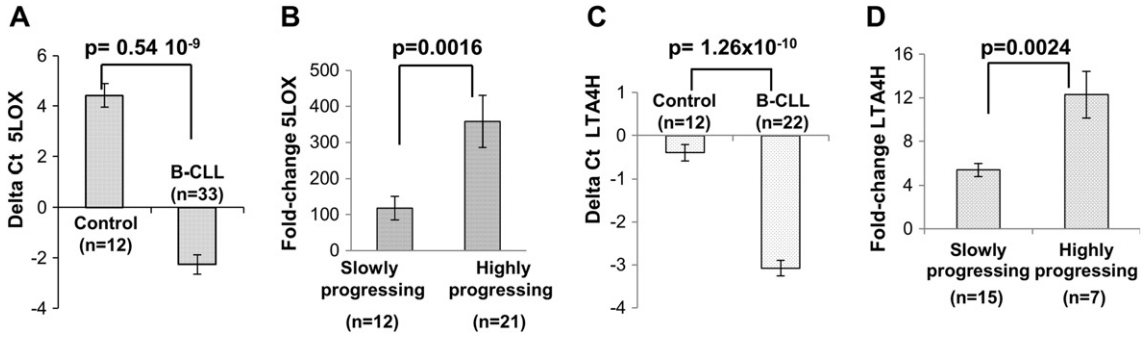

Fig. 3. Analysis of 5LOX and LTA4H expression. A: 5LOX was measured by RT-qPCR using GAPDH as an internal standard (Ct 5LOX–Ct GAPDH) for n = 12 control samples and n = 33 tumor samples. B: Disease progression and 5LOX fold change as measured by RT-qPCR using GAPDH as an internal standard and control cells as reference. C: Expression of LTA4H as measured by RT-qPCR using GAPDH as an internal standard (Ct LTA4H–Ct GAPDH) for n = 12 control samples and n = 22 tumor samples. D: Disease progression and LTA4H fold change as measured by RT-qPCR using GAPDH as an internal standard and control cells as reference. Control stands for healthy B CD5 positive cells (>95% purity as ascertained by flow cytometry).

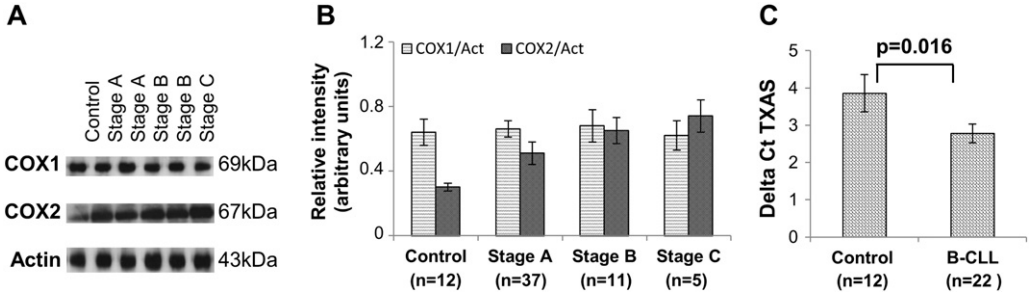

Fig. 4. COX1 and COX2 expression analysis (n = 12 controls, n = 53 patients). A: The Western blot analysis is from a typical analysis B: Mean expression of COX1 and COX2 proteins normalized to actin for n = 12 controls and n = 53 patients (densitometry analysis). C: Expression of TXAS measured by RT-qPCR using GAPDH as an internal standard (Ct TXAS–Ct GAPDH) for n = 12 control samples and n = 22 tumor samples. Control stands for healthy B CD5 positive cells (>95% purity as ascertained by flow cytometry).

5.95 ± 3.51 to 16.91 ± 8.02 (Table 3). For PPAR-alpha, no correlation was found between fold change values and any clinical parameter. By contrast, changes in PPAR-gamma expression correlated with the evolution of the disease. PPAR-gamma was significantly more over-expressed in highly proliferating leukemic cells (n = 13) than in those proliferating slowly (n = 19, p = 0.009, Table 3).

3.6. Inhibition of BLT1, BLT2 and TXAS

We next hypothesized that an autocrine loop might contribute to B-CLL lymphocytic tumor cells survival. In order to analyze the relative contribution of LTB4 through BLT1 and BLT2 binding and of 12-HHT, the high affinity ligand for BLT2 resulting from the action of TXAS on PGH2, we made use of specific inhibitors for 6 healthy B CD5 positive controls, 6 patients with a slowly progressing disease and 6 patients with a highly progressing one. BLT1 and BLT2 cell surface expression, according to the flow cytometry analysis, was respectively 15 ± 5% and 16 ± 7% for the controls, 20 ± 5% and 24 ± 7% for the cells of patients with a slowly progressing disease, and 31 ± 9% and 52 ± 10% for those of patients with an aggressive one. Survival was evaluated using a MTT assay (optical density read at 570 nm). Without any treatment, the optical density was statistically higher for B-CLL cells of patients with an aggressive disease than that of tumor cells of patients with a slowly progressing disease (p = 0.04, Fig. 8). The optical densities for slowly progressing B-CLL and control cells were not distinct from one another (Fig. 8). Adding LTB4 allowed an increase of the optical density for B-CLL cells but not for control cells and this was more important when the disease was aggressive (p = 0.05 for slowly progressing disease, p = 0.01 for highly progressing disease). Inhibiting BLT1 before adding LTB4 resulted in no statistical difference between these two conditions. This was also observed when no LTB4 was added. However, adding the BLT2 inhibitor significantly reduced the value of the optical density of B-CLL for patients with an aggressive disease (p = 0.01) but not for that of control cells or that of B-CLL cells of patients with a slowly progressing disease. Adding a BLT2 inhibitor also reduced the survival of B-CLL cells of patients with adverse evolution but this was not observed for control cells and tumor cells of patients with no unfavorable evolution. No synergy or additivity was observed between BLT1 and BLT2 inhibitors, in any of the conditions tested. Furthermore, inhibition of TXAS with ozagrel did not result in any significant variation of the survival for any of the cell populations tested.

4. Discussion

The mechanisms governing the occurrence and the progression of B-CLL are yet poorly understood. This study allowed us to identify

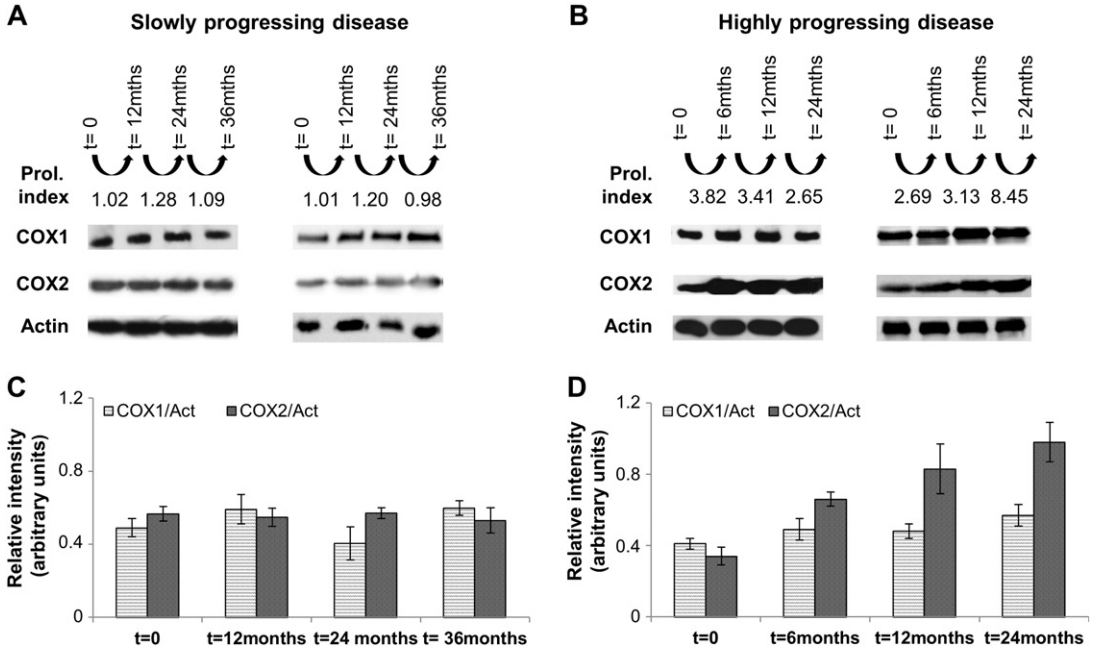

Fig. 5. COX1 and COX2 expression over a 36 months follow-up for slowly progressing disease (A, n = 24) or highly progressing disease (B, n = 19). The Western blot analyses are from typical analyses. C and D: Mean expression normalized to actin for patients with a slowly progressing disease (C, n = 24) or a highly progressing disease (D, n = 19) (densitometry analysis).

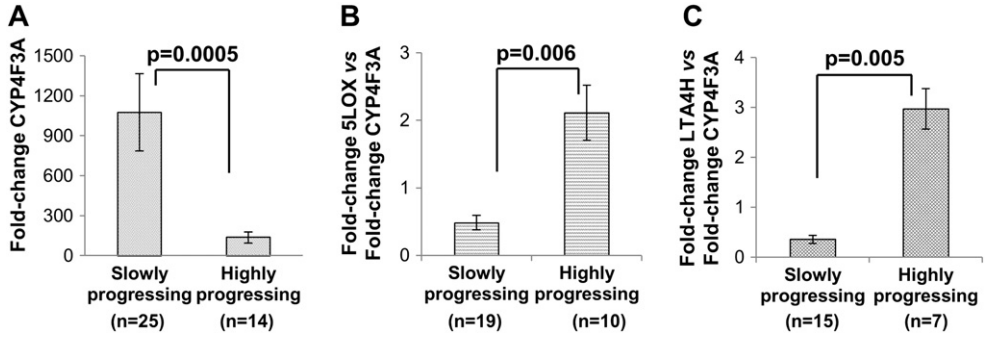

Fig. 6. A: Disease evolution and fold-change in CYP4F3A expression as measured by RT-qPCR. GAPDH was used as an internal standard and healthy B CD5 positive cells as a reference. B: Disease evolution and ratio between fold changes in 5LOX and CYP4F3A expression. C: Disease evolution and ratio between fold changes in LTA4H and CYP4F3A expression.

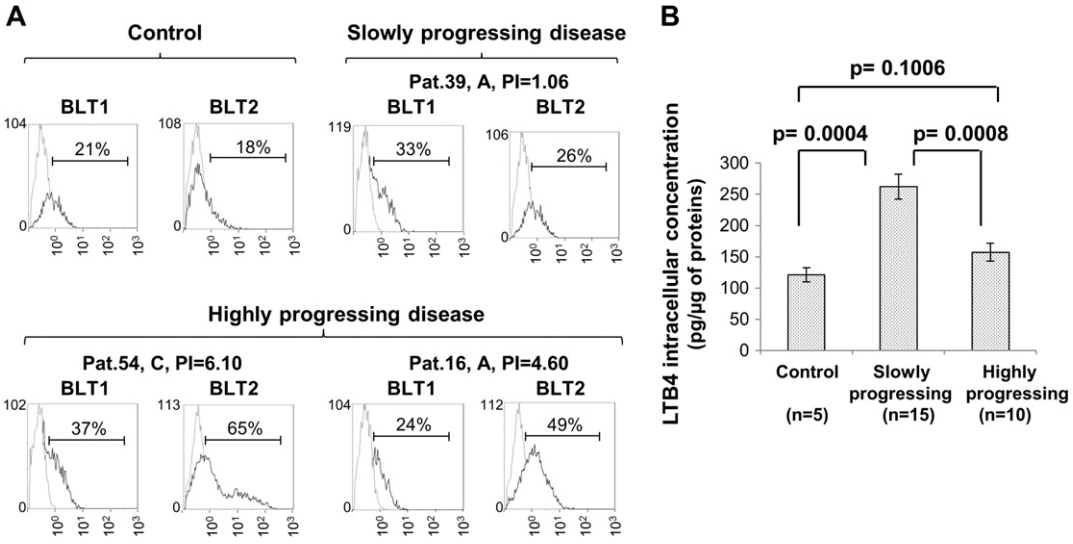

Fig. 7. A: Surface expression of BLT1 and BLT2 receptors as assessed by flow cytometry on at least 5×10^5 purified cells (purity ≥ 95%). Isotypic control is shown as a thin line, BLT1 and 2 staining as bold lines. Pat: Patient; the letter in capital is the stage of the disease at diagnosis, PI: proliferation index. B: Disease evolution and intracellular concentration of LTB4 (pg LTB4/µg of proteins) as measured by ELISA assay.

novel mechanisms of action of lipid mediators on the pathogenicity of B-CLL. Analyzed globally, normal B CD5 positive cells were readily distinguished from B-CLL cells. Indeed, iPLA2-beta, 5LOX, LTA4H, CYP4F3A, TXAS, PPAR-alpha, PPAR-gamma and LTB4 levels were raised in B-CLL cells, in comparison with normal B CD5 positive cells. This suggests that alterations of lipid metabolism may be important events in the etiology of B-CLL. Lipid mediators are known to play important roles in tissue homeostasis by providing energy sources that can fuel in mitochondria metabolism [33]. In addition, they are involved in signaling pathways, some of which may be deregulated in disease [33–35]. They are also important in inflammation, especially the eicosanoids, such as leukotrienes and prostaglandins [34,35]. In this study, we found that COX1, COX2, and TXAS enzymes were abundantly expressed in B-CLL. However, in vitro blockade of TXAS with ozagrel™ resulted in no significant variation of the survival of control B CD5 positive cells and tumor cells of patients with a favorable or an unfavorable clinical outcome. This suggests that 12-HHT is not a major actor in the progression of B-CLL disease. When no addition was made of inhibitors nor LTB4, the optical density observed for the tumor cells of patients with an adverse evolution was superior to that found for the cells of patients without adverse evolution. As equal number of cells were seeded per well and as B-CLL tumor cells do not proliferate in vitro [1,3,4,13], it then seems to us that this difference in optical densities may be attributed to a lesser death rate when the disease is aggressive rather than to a real proliferation. Moreover, as also observed by Secchiero et al. [11], only COX2 was over-expressed in tumor cells, in comparison with normal B CD5 positive lymphocytes. In addition, and in agreement with Ryan's study [10], COX2 over-expression was significantly more important when the proliferative rate was high and thus the disease outcome was poor. These two studies also demonstrated that COX2 over-expression contributes to B-CLL tumor cells' survival and that selective COX2 inhibitors sensitize tumor cells to chlorambucil and TRAIL-induced apoptosis in vitro for most of the patients. It was proposed that COX2 inhibitors may act by modulating B-CLL tumor cells oxidative stress [36,37]. However, administration of the COX2 inhibitor celecoxib did not convincingly improve the patients' condition, even though 3 of the 13 treated-

Table 3
Fold change in PPAR-alpha and PPAR-gamma expressions as measured by RT-qPCR using GAPDH expression as an internal standard and healthy B CD5 positive cells as a reference.

	Stage A (n = 22)	Stage B (n = 7)	Stage C (n = 3)	Slowly evolving	Highly evolving
PPAR-alpha	252.51 ± 47.47	194.61 ± 53.77	207.25 ± 47.96	242.86 ± 49.88	249.24 ± 50.27
PPAR-gamma	6.91 ± 1.82	5.95 ± 3.51	16.91 ± 8.02	6.72 ± 2.28	18.08 ± 8.41

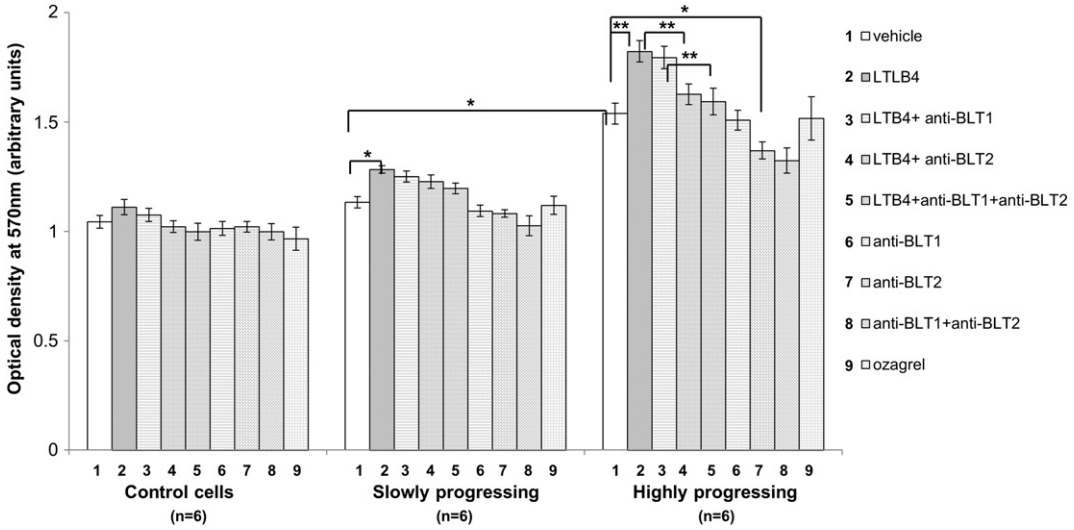

Fig. 8. The LTB4–BLT2, but not the LTB4–BLT1 or the 12-HHT, signaling pathway, is specifically required for the cell survival and proliferation of aggressive B-CLL cells. Cells were incubated with or without 150 nM LTB4 and specific inhibitors. The vehicle was ethanol, BLT1 inhibitor (U78502) was used at the final concentration of 1.5 μM, BLT2 inhibitor (LY255283) was used at 10 μM and the TXAS inhibitor (ozagrel) at 20 μM. * = $p < 0.05$; ** = $p < 0.01$. Surface expression of BLT1 and BLT2 as assessed by flow cytometry was respectively 15 ± 5% and 16 ± 7% for the controls, 20 ± 5% and 24 ± 7% for the cells of patients with a slowly progressing disease, and 31 ± 9% and 52 ± 10% for those of patients with an aggressive disease.

patients showed a greater than 10% decrease in peripheral B-CLL tumor cells count [38]. One reason for this poor efficacy may be the involvement of LTB4 in B-CLL tumor cells' survival. Indeed, these cells were shown to produce LTB4, and specific LTB4 synthesis inhibitors counteracted their CD40-dependent activation [12]. We also observed the production of LTB4 by B-CLL tumor cells. However, plasma concentrations were not statistically different between patients and controls, despite the fact that intracellular B-CLL concentrations of LTB4 were higher than in normal B CD5 positive lymphocytes. In good agreement with this observation, 5LOX and LTA4H mRNAs were more expressed in B-CLL cells than in healthy CD5 positive cells, suggesting that the 5LOX and LTA4H enzymes, which produce LTB4, were more expressed in tumor cells than in normal cells. Why then was there no increase in circulating LTB4 in the patients' serum? One possibility stands from the fact that CYP4F3A, which metabolizes LTB4 into 20-OH-LTB4, a non-secreted compound, was also more abundant in B-CLL tumor cells than in their normal counterparts. Consequently, the balance between synthesis and inactivation may remain roughly constant, at least for the patients whose tumor cells proliferate slowly, less than doubling each year. In addition, 20-OH-LTB4 is known to repress BLT1 expression [15], possibly accounting for the fact that its expression remains close to the one observed in normal B CD5 positive lymphocytes. The situation seems different for patients with a rapidly progressing disease when compared with those with a slowly progressing disease: they show lower intracellular LTB4 concentrations despite lower CYP4F3A and higher 5LOX and LTA4H expression. We therefore analyzed the expression of BLT1 and BLT2, the LTB4 receptors. While BLT1 expression does not vary significantly between the two groups of patients, the expression of BLT2 does, with a larger fraction of expressing cells when B-CLL tumor cells are more proliferative. We then suggest, for those patients, that their tumor cells produce and secrete more LTB4, but that the secreted LTB4 readily binds its membrane receptors BLT1 and BLT2. This may then provide tumor cells with new capacities, depending on the mobilization of either BLT1 or BLT2, which have recently been shown to have non-redundant physiological roles. BLT2 is indeed a low affinity receptor for LTB4 but also a receptor promoting cancer progression by activating the NF-κB transcription factor and the JAK/STAT3 pathway [39,40], which have also been associated with adverse evolution in B-CLL [41,42]. To test this hypothesis, healthy B CD5 positive and tumor cells of patients with a slowly or a highly proliferating disease were incubated with specific inhibitors for these receptors. Blocking BLT1 did not have any effect on the different cellular populations, either in the presence of added LTB4 or not. However, decreased viability was observed for the cells from patients with an aggressive disease when the specific BLT2 antagonist LY255283 was added. This was especially true when LTB4 was added ($p = 0.01$) but was also evidenced when no LTB4 was added ($p = 0.05$). As adding LTB4 resulted in increased survival for both tumor cells of patients with a favorable or an unfavorable clinical outcome, LTB4 trapping on BLT2 thus appears as a mechanism especially involved in the aggressiveness of B-CLL. It then seems that mainly the lipoxygenases, but also the cyclooxygenases, pathways contribute to B-CLL progression. These pathways share a common metabolic precursor, arachidonic acid (AA), whose amount may then be rate limiting for B-CLL proliferation. As intracellular AA is generated from membrane phospholipids, essentially by cPLA2-alpha and iPLA2-beta [6,9], we wondered whether the expression of those enzymes might be altered in B-CLL tumor cells. Normal B CD5 positive lymphocytes and B-CLL express both cPLA2-alpha and iPLA2-beta but at varying levels. Consistent with 5LOX, COX1 and COX2 over-expression, PLA2 enzymes are more present in tumor cells than in their normal counterparts. While iPLA2-beta expression does not appear to be linked with the tumor stage, cPLA2-alpha is more expressed in tumor cells when patients suffer from an advanced disease (stages B and C according to Binet classification). However, of interest is the fact that over-expression of both PLA2s correlates with a high proliferative index, as also observed for 5LOX, LTA4H, COX1 and COX2. This may be relevant to the fact that both iPLA2-beta and cPLA2-alpha have been proposed to promote cancer progression by enhancing cell migration or cell proliferation [6,43–46], on the condition that AA intracellular concentration remains low, otherwise free AA is cytotoxic and promotes cell death [47]. Our findings are consistent with these physiological roles for iPLA2-beta and cPLA2-alpha.

LTB4 and its metabolite 20-OH-LTB4, prostaglandins and their metabolites are bioactive lipids, which bind and activate the nuclear receptors and transcription factors PPAR-alpha and PPAR-gamma. In turn, these regulate their synthesis and degradation, as demonstrated for LTB4 and PPAR-alpha [9,19,21–23]. In our study, both receptors were more expressed in circulating B-CLL tumor cells than in normal B CD5 positive lymphocytes. PPAR-gamma was significantly more abundant in tumor cells with a high proliferative index than in slowly growing ones. This might seem surprising as PPAR-gamma is considered as a tumor suppressor gene, although its main function relates to its role in lipid metabolism [7,25,48]. Identifying PPAR-gamma target genes in

B-CLL may then be of interest to fully understand its role in this disease. Spaner et al. [49] recently demonstrated PPAR-alpha expression in circulating B-CLL cells, as we did here. However, contrary to their results, we found PPAR-alpha to be moderately expressed in normal B CD5 positive cells, but much more strongly in all B-CLL tumor cells, not only in advanced stage disease. Discrepancy between the two studies may relate to the techniques used. In Spaner's study, PPAR-alpha levels were measured by Western-blotting for 26 B-CLL samples and RNA quantitation for only 2 B-CLL samples. In the last cases, PPAR-alpha expression was moreover compared with that observed in the mononuclear cells from a single normal donor. Nevertheless, both studies, together with that of Tung [50], are consistent with a putative role for PPAR-alpha in the course of tumor progression. The ability of PPAR-alpha and PPAR-gamma to allow fatty acids to be used as a source of energy may surpass their potential tumor suppressor function.

5. Conclusion

Taken together our results demonstrate a deregulation of the pathways leading to LTB4 and prostaglandins synthesis. These alterations affect the first elements common to these pathways: cPLA2-alpha and iPLA2-beta, together with 5LOX, LTA4H, COX1, COX2 and TXAS. This starts from the early stage and is being amplified for tumor cells with a high proliferative capacity, except for COX1 and TXAS. As a consequence, prostaglandins and LTB4 can be synthesized, BLT1 and BLT2 activated, thus allowing tumors to increase their survival and proliferative potentials. In parallel, PPAR-alpha and PPAR-gamma seem to function more as cancer promoting transcription factors rather than tumor suppressor genes, enabling tumor cells to use fatty acids as an energy source. The combined use of COX and LOX inhibitors may then be of potential clinical interest in B-CLL treatment.

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