Thioredoxin, produced by stromal cells retrieved from the lymph node microenvironment, rescues chronic lymphocytic leukemia cells from apoptosis *in vitro*

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Funding: this work was supported by funding provided by the Östergötland County Cancer Research Fund and Swedish Cancer Society 3171-B04. GSD.

Acknowledgments: we thank Drs. Bernt Boeryd, Claes Malm and Franz Rommel, and Ms Annika Persson for help with the clinical specimens from the patients with CLL. We also thank Simin Tahmasebpoor for help with sectioning the lymph node tissues.

Manuscript received February 27, 2007
Manuscript accepted September 1, 2007

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ABSTRACT

Background and Objectives

The redox-regulatory protein thioredoxin has several functions including transcriptional regulation, and antioxidant, cytokine, and chemokine activities. We have previously shown that extracellular thioredoxin protects B-cell chronic lymphocytic leukemia (CLL) cells from apoptosis *in vitro*. In this study we were interested to determine whether thioredoxin is produced by cells surrounding the CLL cells in the *in vivo* microenvironment and whether this cell-derived thioredoxin has any leukemia growth-promoting effect *in vitro*.

Design and Methods

Lymph nodes from CLL patients (n=25) were analyzed for thioredoxin expression by immunohistology. Stromal cells purified from the lymph nodes were analyzed for thioredoxin secretion at the single cell level using an ELIspot assay. The survival effect of the stromal-derived thioredoxin was tested by co-culturing stromal- and CLL cells with and without Fab-fragments of an anti-thioredoxin antibody.

Results

The results indicated that the thioredoxin production correlated with the amount of proliferating cells and was mainly localized to the proliferation centers (pseudofollicles) in the CLL lymph nodes. The leukemia cells *per se* showed minimal thioredoxin levels; in contrast, stromal cells strongly expressed thioredoxin. Purified primary stromal cells, which secreted extracellular thioredoxin, significantly protected the CLL cells from undergoing apoptosis in 72 h co-cultures. Interestingly, this anti-apoptotic effect could be abrogated by addition of Fab-fragments of an anti- thioredoxin antibody.

Interpretation and Conclusions

In conclusion, we have shown that stromal cells in the lymph node microenvironment produce thioredoxin and that the thioredoxin production is localized to the proliferation centers of the CLL lymph nodes. In addition, thioredoxin produced by purified stromal cells rescued CLL cells from apoptosis *in vitro*.

Key words: CLL, thioredoxin, stromal cells, microenvironment.

Haematologica 2007; 92:1495-1504. DOI: 10.3324/haematol.11448

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B-cell chronic lymphocytic leukemia (CLL) is characterized by increasing high numbers of long-lived, monoclonal CD5+ B cells in bone marrow, blood and secondary lymphoid tissue.[1] Nowadays, it is generally accepted that the resistance to programmed cell death and the selective survival advantage of CLL cells is not only an autonomous characteristic, but depends on external anti-apoptotic stimuli. This is supported by the fact that despite their longevity *in vivo*, CLL cells often undergo spontaneous apoptosis under *in vitro* growth conditions. This also implies that *in vitro* cultured CLL cells lack essential survival signals that are present in the *in vivo* microenvironment.

Although the CLL cells in the blood are regarded as non-dividing, a small fraction of CLL cells replicate in proliferation centers (pseudofollicles) in bone marrow and lymph nodes.[2,3] Recently, it was shown that the birth rate of CLL cells varies from 0.1% to greater than 1% per clone and day.[4] In addition, several cases of CLL with atypical morphological features in the bone marrow have, besides quiescent and proliferating cells, also subpopulations of CLL cells that undergo spontaneous apoptosis.[5] This implies that the disease process is more dynamic than previously considered and that it is characterized by proliferating as well as dying cells. Selected microenvironmental signals delivered by accessory cells, such as bone marrow stromal cells and T cells appear to confer CLL cells with a growth advantage and extended survival.[6,7] To further identify important growth-promoting molecular interactions, this study focused on the redox-active thioredoxin protein. Thioredoxin is a multifunctional protein, ubiquitously expressed at a low level in all cells of the body. Intracellular thioredoxin has anti-apoptotic as well as growth-promoting effects and additionally, some types of cells have the capacity to release thioredoxin. This extracellular form of thioredoxin has cytokine and chemokine activities.[8] Interestingly, exogenous thioredoxin was previously shown to protect malignant CLL cells from apoptosis *in vitro*.[9]

In this study, the potential *in vivo* role of thioredoxin was addressed by analyzing this protein's expression in CLL lymph nodes. Furthermore, lymph node-derived stromal cells including fibroblastic reticular cells (FRC) and follicular dendritic cells (FDC) were isolated and analyzed for thioredoxin secretion and the ability to protect CLL cells from apoptosis.

Design and Methods

Patients' samples

This study was approved by the ethical committe of Linköping University (no. 00-111 and no. M30-05). Lymph nodes were collected from 13 patients with CLL (ID-1 to ID-13) and peripheral blood was collected from five CLL patients (ID-11; ID-13 to ID-16). The CLL patients were selected among consecutively attending patients at the Haematology Clinic, University Hospital, Linköping, Sweden. In addition, lymph node sections from 12 CLL patients (ID-17 to ID28) were collected at the Department of Pathology, Uppsala University Hospital, Uppsala, Sweden. Morphologic classification was performed according to the World Health Organization (WHO) classification and the tumors typically expressed CD5, CD23 and showed weak expression of Ig. Table 1 shows the clinical characteristics of the patients. Their *IGHV* mutational status and CD38 expression have been published previously.[10,11] Control tonsils were retrieved from children undergoing tonsillectomy because of sleeping problems.

Immunohistology

Formalin-fixed, paraffin-embedded sections from ten CLL lymph nodes (ID-1 to ID-10) and control lymph nodes (tonsils) were analyzed by immunohistochemistry. The tissues were submerged in 0.01 M Tris-EDTA, pH 9 at 94°C for 30 min for antigen retrieval and thereafter stained with the antibodies described in Table 2. Mayer´s hematoxylin (Histolab, Göteborg, Sweden) was used for counterstaining and Mount Quick Aqueous (Histolab) for mounting. A Nikon microscope ECLIPSE E600W and Nikon digital camera DXM1200F together with the ACT-1 application program were used for imaging (Nikon, Badhoevedorp, The Netherlands). For subsequent analyses Adobe Photoshop CS2 9.0 software was applied (Adobe Systems, Mountain View, CA, USA).

For immunofluorescence 15 CLL lymph nodes (ID-11 to ID-13 and ID-17 to ID-28) were snap-frozen in pre-cooled isopentane and stored at -80°C until sectioning. Cryostat sections of 6 μm were sliced and placed on room temperature glass slides. The slides were then allowed to dry for at least 1 h at room temperature before fixation in acetone for 10 min and then dried for 15 min at room temperature. The tissues were incubated with the antibodies described in Table 2. SlowFade Gold mounting medium with DAPI (Molecular Probes) was used for mounting. A Zeiss Axiovert 200M inverted microscope, equipped with an AxioCam MRm camera and a Carl Zeiss 63×, N.A. 1.2, water objective, was employed for image acquisition (Brattleboro, VT, USA). Images were collected and analyzed using Zeiss AxioVision version 3.1 software. For subsequent analyses Adobe Photoshop version CS2 9.0 software was used (Adobe Systems, Mountain View, CA, USA). To study correlations, the stained area of the tissue sections stained for Ki-67, thioredoxin and FDC was quantified, using the Image J 1.37v image analyzing program from the NIH, as a proportion of the total area. Mean values are based on measurements from three to six fields for each patient, depending on the size of the lymph node sections To analyze thioredoxin secretion from purified accessory stromal cells, cells were seeded onto glass

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Table 1. Clinical characteristics of the CLL patients.

Pt.	Age	Sex*	Stage (Rai)	Time from diagnosis to sample collection (year of diagnosis)†	Treatment†	Mutation status‡	$CD38^+$ cells %
1	81	M	II	1 (1997)	Untreated	NA	NA
2	79	M	II	0 (2000)	Untreated	NA	NA
3	67	M	III	0 (1999)	Untreated	NA	NA
4	78	F	I	0 (1996)	Untreated	NA	NA
5	82	M	III	0 (1995)	Untreated	NA	NA
6	59	M	0	3 (1992)	Untreated	UM	NA
7	67	M	II	1 (1997)	Untreated	NA	NA
8	68	M	I	0 (2001)	Untreated	NA	NA
9	58	M	II	0 (2001)	Untreated	M	NA
10	54	M	IV	0 (2001)	Untreated	UM	NA
11	58	M	II	4 (2001)	Treated (2002)	M	NA
12	64	M	I	8 (1997)	Treated (1998)	UM	NA
13	58	M	IV	1 (2005)	Untreated	UM	NA
14	81	F	I	0 (2005)	Untreated	UM	NA
15	63	F	II	3 (2002)	Treated (2002)	NA	NA
16	65	M	IV	11 (1994)	Treated (2002)	UM	NA
17	72	M	I	2 (1993)	Untreated	M	80
18	59	F	I	0 (1993)	NA	M	15
19	77	M	I	0 (1993)	Untreated	M	NA
20	79	M	I	0 (1990)	Untreated	UM	70
21	73	M	I	0 (1997)	Untreated	UM	5
22	46	M	IV	0 (1998)	Untreated	UM	70
23	78	M	I	0 (1988)	Untreated	UM	NA
24	73	M	I	2 (1981)	Treated (1983)	M	85
25	55	M	I	15 (1983)	Untreated	UM	51
26	68	F	I	0 (1996)	Untreated	UM	75
27	61	F	I	0 (1992)	Untreated	UM	9.8
28	53	M	II	0 (1995)	Untreated	UM	85

*M: male; F: female; †Year;up to sample date; ‡M: mutated; UM: unmutated; NA: No clinical data available.

slides and incubated at 37°C in humidified air containing 5% CO_2. The cells were fixed in 4% ice cold paraformaldehyde for 5 min and treated with 0.1% saponin for permeabilization. Thereafter, the cells were stained using the same procedure as for the lymph nodes tissues.

Cell purification and phenotype analysis

The cells used in this study were cultured in RPMI 1640 medium supplemented with 45 μg/mL penicillin, 45 μg/mL streptomycin, 1.8 mM L-glutamine and 10% fetal bovine serum (FBS) (Gibco, Paisley, Scotland) and grown at 37°C in humidified air containing 5% CO_2. Peripheral blood mononuclear cells (PBMC) were purified from blood of CLL patients by centrifugation over a Ficoll-Hypaque density gradient medium (Amersham Pharmacia Biotech, Uppsala, Sweden). All CLL PBMC samples examined contained more than 90% of CLL cells as determined by flow cytometry with anti-CD5-RPE and anti-CD19-RPE-Cy5. Stromal cells including FRC and FDC were purified from CLL lymph nodes (ID-11 to ID-13) and tonsil tissue. Single cell suspensions were retrieved after mincing the lymph nodes and tonsils in a stainless steel mesh. The cells were seeded in tissue flasks and adherent cells were fed every fourth day by replacing 50% of the medium until confluence. The phenotype of the adherent cells was analyzed by flow cytometry using the antibodies described in Table 2. Goat (F(ab´)₂) anti-mouse Ig-F (Dako) was used as the secondary reagent for detection of unconjugated antibodies. Flow cytometric analysis was performed on a FACS Calibur Flow Cytometer equipped with CellQuest-Pro research software (Becton Dickinson).

ELISpot and ELISA

The ELISpot assay was performed according to Sahaf *et al.*[12] using the goat anti-thioredoxin polyclonal antibody (A.R.) to coat PVDF-bottomed 96-well microtiter plates (Millipore, Bedford, MA, USA). The biotinylated anti-thioredoxin monoclonal antibody (clone 2G11, IMCO Corporation Ltd. AB, USA) was used as the secondary (detector) antibody. The monocytes used as the positive control were purified as previously described.[12] One hundred thousand stromal cells or monocytes were added per well. The number of spots was counted blindly and independently in a Nikon stereo microscope (35× magnification) by two people. The enzyme linked immunosorbent assay (ELISA) was performed according to Söderberg *et al.*[13] using anti-thioredoxin monoclonal antibody (clone 2G11) as the capture antibody and goat anti-thioredoxin-biotinylated (A.R.) as the indicator antibody.

Survival assay

Stromal cells purified from normal tonsil and CLL lymph nodes were seeded separately in 96-well plates (Corning Inc. NY, NY, USA) at a cell density of 5×10^3 cells/well in R10 medium. The stromal cells were cultured at 37°C in a 5% CO_2 humidified atmosphere for 24 h. Thereafter, the medium was aspirated from the adherent cells and CLL cells were added to the plates at a cell density of 2.5×10^5 cells/well in RPMI supplemented with 45 U/mL penicillin, 45 μg/mL streptomycin, 1.8 mM L-glutamine and 1% FBS (Gibco) in the presence or absence of Fab fragments of anti-thioredoxin (clone 2G11) or control Fab fragments of mouse IgG (Jackson ImmunoResearch Laboratories, Baltimore, PA, USA). Fab fragments of anti- thioredoxin were produced using the ImmunoPure Fab preparation kit (Piece, Rockford, USA). As a control, CLL cells alone were used. After coculturing for 72 h the cells were harvested, and the viability (annexin V-fluoroscein isothiocyanate negative) of the tumor cells ($CD5^+CD19^+$) was analyzed by flow cytometry. Flow cytometric analyses were performed on a Calibur Flow Cytometer equipped with CellQuest-Pro (Becton Dickinson). The viability of the CLL cells was at least 90% at the start of the experiments.

Statistics

Statistical analyses were performed using Student's t-test in Microsoft Excel and Pearson's correlation in GraphPad Prism version 4.00 for Windows. p values <0.05 were considered statistically significant.

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Results

Thioredoxin expression in CLL lymph nodes and tonsils

Our previous study on the role of thioredoxin and its anti-apoptotic effect on CLL tumor cells *in vitro* was extended in this study by analyses of thioredoxin expression in sections of lymph nodes from CLL patients. Thioredoxin was expressed in all lymph nodes specimens although the intensity and frequency of positive cells varied (Figure 1). Low thioredoxin expression could be detected in the CLL tumor cells *per se*. In contrast, thioredoxin was highly expressed in cells with macrophage- or fibroblast-/dendritic-like morphology. To analyze thioredoxin expression in control lymphoid tissue, tonsil sections from tonsillectomized children were stained for thioredoxin. Fibroblast/dendritic-like cells outside the follicles of the cortex and dendritic-/macrophage-like cells inside the follicles showed thioredoxin expression that was significantly stronger than elsewhere in tonsils. The sections were negative for the isotype control mouse IgG (*data not shown*).

Fibroblast reticular cells and follicular dendritic cells overexpressed thioredoxin

In order to identify which cell type(s) were thioredoxin positive in CLL lymph nodes, we applied markers for FRC, FDC, macrophages, T cells and B cells. Thioredoxin was analyzed in frozen lymph node tissue sections from 15 CLL patients (ID-11 to ID-28) by double immunofluorescence using anti-thioredoxin and cell-type specific monoclonal antibodies. The results showed that FRC and FDC, representing stromal cells, expressed thioredoxin strongly (Figure 2A-F), whereas CLL cells and T cells had minimal levels of thioredoxin (Figure 2J-O). Some of the macrophages were also positive for thioredoxin expression (Figure 2G-I). The isotype controls mouse IgG and mouse IgM were negative (Figure 2P-Q).

The expression of thioredoxin correlated with the expression of Ki-67 and FDC

To determine whether the expression of thioredoxin correlated with the expression of Ki-67 (a nuclear proliferation associated protein, absent in resting G_0 cells) and the presence of FDC, lymph nodes from 12 CLL patients (ID-17 to ID-28) were analyzed by immunofluorescence. The mean value (from three to six fields/patient) of the percentage thioredoxin stained area was compared with the mean value (from three to six fields/patient) of the percentage Ki-67 stained area. The results showed that expression of thioredoxin correlated with the expression of Ki-67 (Figure 3A). In addition, in those patients with a high proportion of proliferating cells (Ki-67$^+$) the thioredoxin expression was mainly localized to the proliferation centers (Figure 1, lowest

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Table 2. Summary of antibodies used for immunohistology and flow cytometry.

Primary antibodies	*Type*	*Clone*	*Source*	*Secondary antibodies*	*Source*
Anti-FDC	Mouse IgM	CNA.42	Dako	Goat anti-mouse IgM-Alexa594	MB
Anti-FDC	Mouse IgM	CNA.42	Dako	Goat anti-mouse Ig F(ab´)2-F	
Anti-fibroblast	Mouse IgG	d-7FIB	Acris	Goat anti-mouse IgG F(ab´)2-Alexa488	MB
Anti-fibroblast	Mouse IgG	d-7FIB	Acris	Goat anti-mouse Ig F(ab´)2-F	
Anti-CD68	Mouse IgG	KP1	Dako	Goat anti-mouse IgG F(ab´)2-Alexa488	MB
Anti-CD19	Mouse IgG	HD37	Dako	Goat anti-mouse IgG F(ab´)2-Alexa488	MB
Anti-CD19-RPE-Cy5	Mouse IgG	HD37	Dako		
Anti-CD3-F	Mouse IgG	UCHT-1	BD		
Anti-CD44-F	Mouse IgG	B-F24	Diaclone		
Anti-CD5-F	Mouse IgG	DK23	Dako		
Anti-CD5-RPE	Mouse IgG	DK23	Dako		
Anti-CD14-RPE	Mouse IgG	TUK4	Dako		
Anti-HLA-DR,DQ,DP	Mouse IgG	CR3/43	Dako	Goat anti-mouse Ig F(ab´)2-F	
Anti-CD40-F	Mouse IgG	2C6	Mabtech		
Anti-CD44-F	Mouse IgG	B-F24	Diaclone		
Anti-DC-LAMP	Mouse IgM	104.G4	Immunotech	Goat anti-mouse Ig F(ab´)2-F	
Anti-Ki-67	Mouse IgG	MIB-1	Dako	Envision AP	Dako
Anti-Ki-67	Mouse IgG	MIB-1	Dako	Goat anti-mouse IgG F(ab´)2-Alexa488	MB
Anti-thioredoxin	Mouse IgG	2G11	A.R	Anti-mouse Ig-HRP	Dako
Anti-thioredoxin-F	Mouse IgG	2G11	A.R	Anti-F-HRP	Dako
Anti-thioredoxin-Alexa594	Mouse IgG	2G11	A.R		
Isotype control	Mouse IgM		Dako	Goat anti-mouse IgM-Alexa488	MB
Isotype control	Mouse IgG		Dako	Goat anti-mouse IgG F(ab´)2-Alexa488	MB
Isotype control	Mouse IgG		Dako	Envision AP	Dako
Isotype control	Mouse IgG		Dako	Anti-mouse Ig-HRP	
Isotype control	Mouse IgG-F		Dako		
Isotype control	Mouse IgG-F		Dako	Anti-F-HRP	Dako

Dako,Glostrup, Denmark; Acris, Hiddenhausen, Germany; Beckton Dickinson (BD), San José, CA, USA; Molecular Probes (MB), Carlsbad, CA, USA; Diaclone Research/Tepnel Lifecodes Co; Immunotech, Marseille, France; Mabtech, Stockholm, Sweden.

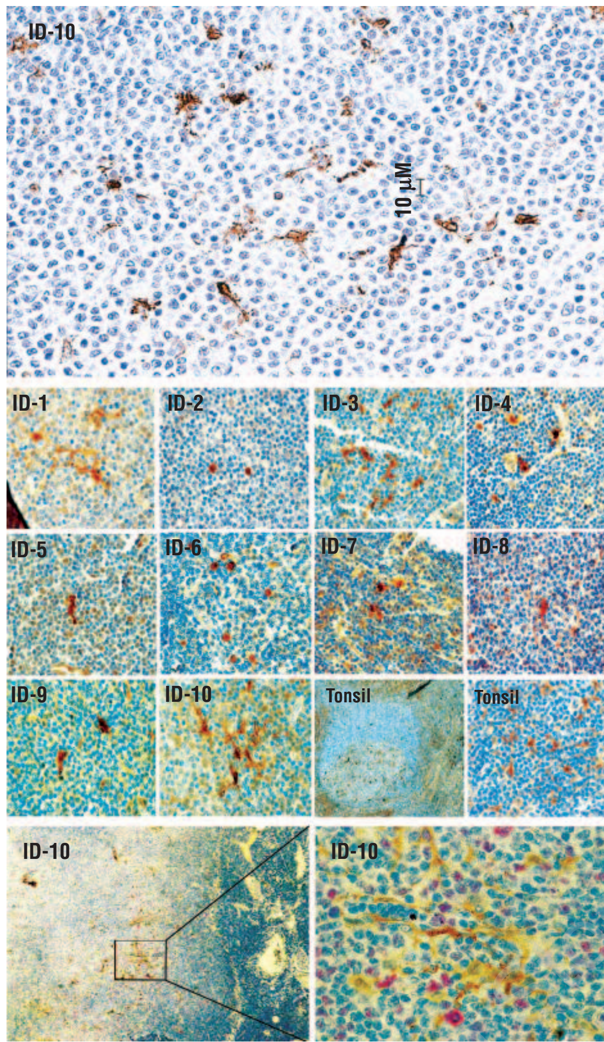

Figure 1. Immunohistochemical detection of thioredoxin and Ki-67 in lymph nodes from CLL patients. Thioredoxin localization in ten different CLL lymph nodes (patient ID-1 to ID-10) and a tonsil section is shown. In single staining thioredoxin was detected with the 2G11 monoclonal antibody, anti-mouse Ig-HRP and DAB. Magnification 600×. Thioredoxin expression in tonsil is shown at 600× magnification as well as at 100× magnification. Double staining of thioredoxin and Ki-67 is shown (at 40× and 600× magnification) in the bottom row. For double staining the tissue was first incubated with Ki-67 monoclonal antibody (clone MIB-1) and developed with Envision AP. Thereafter the tissue was blocked with 5% mouse and 5% rabbit serum before anti- thioredoxin-FITC, HRP-conjugated anti-FITC was added. Fast Red was used as the AP substrate and DAB as the HRP substrate. Mayer´s hematoxylin was used for counterstaining

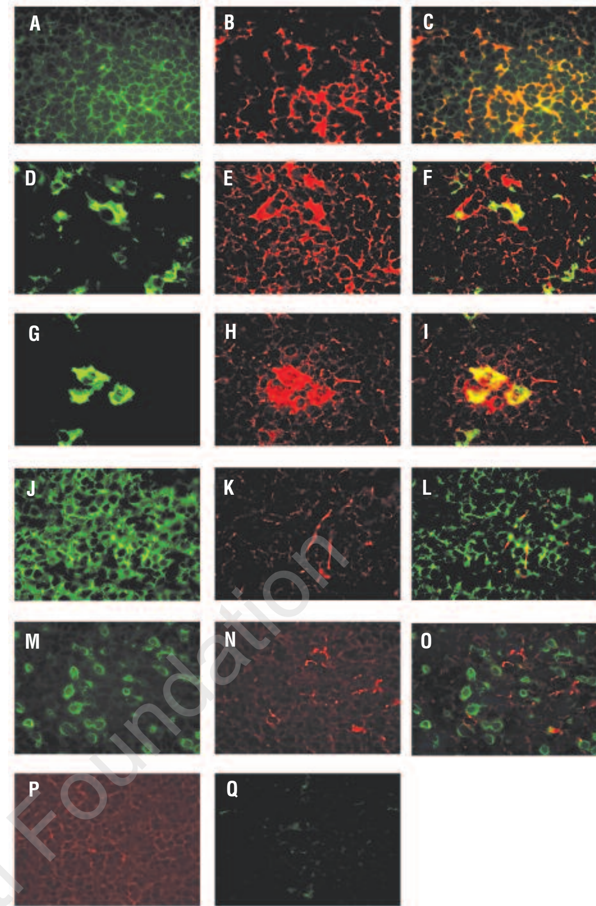

Figure 2. Thioredoxin localization in FRC and FDC from frozen CLL lymph nodes sections. Anti-FDC was developed with Alexa594-conjugated anti-mouse IgM and the staining thus shows as red (A). Co-localization of anti-FDC and thioredoxin is shown as yellow. (B). Biotin-conjugated mouse anti-thioredoxin and Alexa488-conjugated-Streptavidin is shown as green. (C). Anti-FRC (mouse IgG1, clone d-7FIB), anti-CD68 (IgG1) and anti-CD19 (IgG1) was developed with Alexa488-conjugated anti-mouse IgG, $F(ab')_2$ and the staining is thus shown as green (D, G and J, respectively). Alexa594-conjugated anti-thioredoxin staining is shown as red (E, H and K). Co-localization of the FRC, CD68 and CD19 markers and thioredoxin is shown as yellow (F, I and L, respectively). Anti-CD3-FITC staining is shown as green (M) and Alexa594-conjugated anti- thioredoxin staining in red (N). Double staining is shown in O. The isotype controls mouse IgM together with Alexa594-conjugated goat anti-mouse IgM (P) and mouse IgG together with Alexa488-conjugated goat anti-mouse IgG, $F(ab')_2$ (Q) is also shown. 630× magnification. Immunofluorescence staining of a sample from one representative CLL patient (ID-12) out of three (ID-11 to ID-13) is shown.

panel). The Ki-67$^+$ cells in the proliferating centers were confirmed to be mainly CD5$^+$CD19$^+$ leukemic cells (*data not shown*). The thioredoxin expression also correlated with the presence of FDC (Figure 3B) and, as shown in Figure 2 A-C, the thioredoxin expression was co-localized to the FDC. There was no significant difference in thioredoxin expression between unmutated cases and mutated cases (p=0.16) or between CD38$^+$ and CD38$^-$ cases (p=0.18). Because ten of the 12 patients had stage I CLL the correlation of thioredoxin with Rai stage was not analyzed.

Stromal cell-derived thioredoxin rescued CLL cells from apoptosis

Adherent cells with fibroblast-/dendritic-like morphology were purified from tonsils or CLL lymph nodes and phenotyped by flow cytometry. CD5 and CD19 were not expressed by the purified adherent cells excluding T- and B-cells (Figure 4 and Table 3). Furthermore, none of the cells expressed CD14, HLA-DR, -DP, -DQ or DC-LAMP, indicating absence of monocytes/macrophages and interdigitating dendritic cells (Figure 4 and Table 3). More than 90% of the adher-

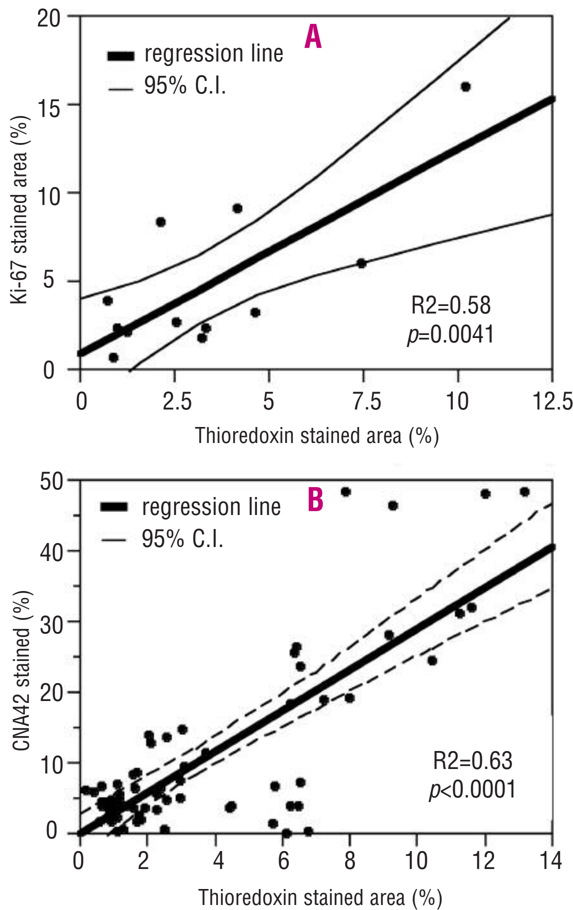

Figure 3. The overall thioredoxin expression in CLL lymph nodes correlated with the expression of FDC and Ki-67. The expression of thioredoxin and proliferating cells (Ki-67) was analyzed by single color immunofluorescence on successive sections as stained area as a proportion of the total area (A). Mean values from three to six fields/patient is shown (A). The expression of thioredoxin and FDC was analyzed by two color immunofluorescence on the same section (B). The mean values from three to six fields for each patient are shown (B). Pearson´s correlation test was used for statistical analysis of the results from 12 patients (ID-17 to ID-28).

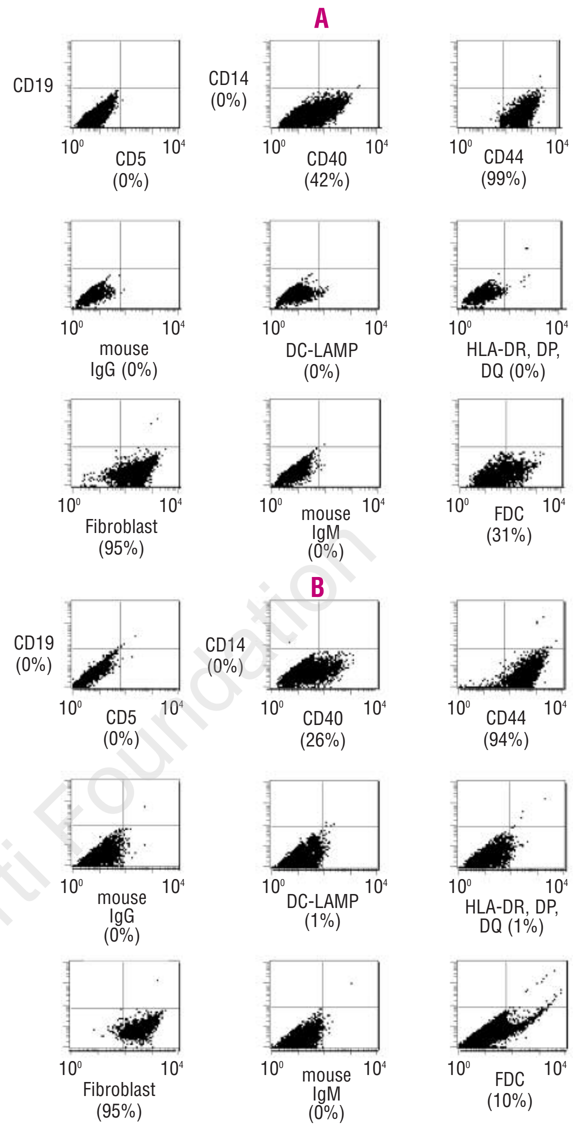

Figure 4. Stromal cell-like phenotype of the purified adherent cells from tonsils and CLL lymph nodes. Adherent cells purified from tonsils (A) or CLL lymph nodes (B) were stained with anti-CD5-F (clone DK23), anti-CD19-RPE (clone HD37), anti-CD40-F (clone 2C6), anti-CD14-RPE (clone TUK14), anti-CD44-F (B-F24), anti-DC-LAMP (clone 104.G4), anti-HLA-DR,DP,DQ (clone CR3/43), anti-fibroblast (clone d-7FIB), and anti-FDC (clone CNA.42). Goat (F(ab´)₂) anti-mouse Ig-F was used to detect the unconjugated antibodies. Mouse IgG and mouse IgM were used as isotype controls. The percentages of positive cells are shown below the labeled axes. One representative experiment out of the three included in Table 3 is shown.

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ent cells from CLL lymph nodes, as well as from tonsils, expressed CD44 and the fibroblastic marker d-7FIB (Figure 4 and Table 3). Stromal cells from tonsil and/or bone marrow have previously been shown to express CD44 and the fibroblastic marker.[14, 15] A subpopulation of the cells from the tonsil as well as the CLL lymph nodes expressed CD40 and the FDC marker CNA.42 (Figure 4 and Table 3). Thus, the fibroblast-/dendritic-cell morphology and the presence of fibroblastic and FDC markers revealed the cells to be stromal cells including FRC and FDC. The stromal cells were analyzed for expression and secretion of thioredoxin by immunofluorescence and ELISpot assay, respectively. For immunofluorescence, the cells were double-stained with anti-CD44 (both FRC and FDC were CD44$^+$) and anti-thioredoxin monoclonal antibody. The results showed that all cells contained thioredoxin (Figure 5A). The ELISpot technique was used for detection of thioredoxin-secretion at the single cell level. As shown in Figure 5B, some of the stromal cells were potent thioredoxin-secretors. Monocytes from normal blood donors, previously shown to secrete thioredoxin,[12] were used as a positive control. No false positive spots were observed in controls for necrotic cell-release using detergent-lyzed cells (Figure 5B). In contrast to small round spots generated by monocytes secreting thioredoxin, the spots generated by stromal cells were large, which reflects the morphology of fibroblast and dendritic cells (*data not shown*). In addition to the ELISpot assay, culture super-

Table 3. Phenotype of adherent cells purified from tonsils and CLL lymph nodes.*

Antigen (monoclonal antibody clone)	*Tonsil*	*CLL lymph node*
CD5 (DK23)	0±0	0±0
CD19 (HD37)	0±0	0±0
CD14 (TUK14)	2±2	2±1
DC-LAMP (104.G4)	0±0	2±2
HLA-DR,DP,DQ (CR3/43)	0±0	1±1
CD40 (2C6)	37±14	40±12
CD44 (B-F24)	96±5	98±3
Fibroblast (d-7FIB)	98±3	97±2
FDC (CNA.42)	26±5	13±3
Mouse IgG	0±0	0±0
Mouse IgM	0±0	0±0

**Percentage of positive cells as analyzed by flow cytometry. The mean values ± SD of cultures from three distinct CLL lymph nodes and tonsils are shown.*

natants were analyzed in ELISA, confirming thioredoxin-secretion by the stromal cells in the range of 10-50 ng/mL/72 h/2.5×10^5 cells. The ELISpot, ELISA and immunofluorescence assays are complementary since they measure slightly different aspects (level of response, dynamic range and intervals of concentration, etc.); ELISpot is better suited for low level responses.[16] However, since all stromal cells expressed thioredoxin, but only a proportion of these cells secreted thioredoxin, it cannot be excluded that there is/are subpopulation(s) among the purified stromal cells that are responsible for the thioredoxin secretion.

To analyze whether isolated stromal cells, shown to secrete thioredoxin *ex vivo*, had any effect on CLL cell survival, leukemia cells were cultured with or without adherent stromal cells. We found that stromal cells, retrieved either from tonsils or CLL lymph nodes, significantly reduced the percentage of apoptotic tumor cells in 72 h co-cultures (p=0.006 and p=0.005, respectively) (Figure 6A). There was no difference in anti-apoptotic effect when co-cultures of CLL cells and autologous stromal cells were compared with co-cultures of CLL cells and stromal cells from different patients (*data not shown*). The stromal cells did not express MHC class II, thus making allogeneic responses unlikely. The frequency of apoptotic CLL cells after co-culture with stromal cells differed to some extent depending on the CLL donor. However, in all experiments performed, there was a significant increase of viable cells in co-cultures of CLL and stromal cells compared with cultures of CLL cells alone.

The addition of Fab fragments of an IgG anti- thioredoxin monoclonal antibody to the CLL and stromal cell co-cultures significantly abrogated the anti-apoptotic effect mediated by the stromal cells (p=0.0018) (Figure 6B and 7). This increase could not be blocked by Fab fragments of mouse control IgG (p=0.8206) (Figure 6B and 7). No significant effects on cell survival were recorded when anti- thioredoxin monoclonal antibody was added to CLL cells alone (p=0.0700) (Figure 6 and 7). The increase in frequency of apoptotic CLL cells was dependent on the concentration of the anti-thioredoxin Fab fragments (Figure 6C). As shown in Figure 6B, addition of 20 μg/mL anti-thioredoxin Fab fragments was sufficient to significantly block the improved survival of CLL cells mediated by the stromal cells.

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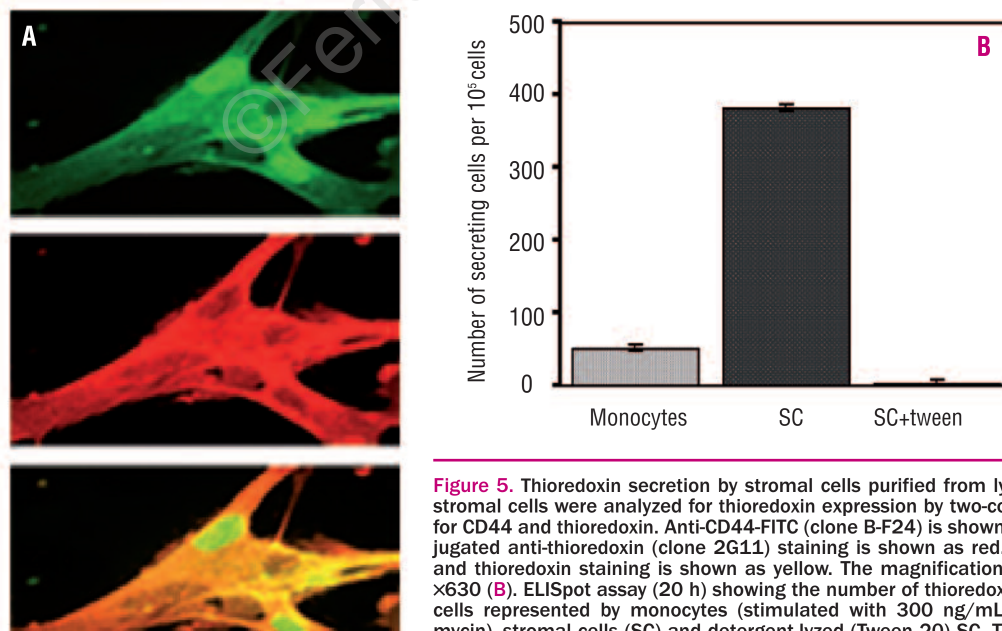

Figure 5. Thioredoxin secretion by stromal cells purified from lymph nodes (A). Isolated stromal cells were analyzed for thioredoxin expression by two-color immunofluorescence for CD44 and thioredoxin. Anti-CD44-FITC (clone B-F24) is shown as green. Alexa594-conjugated anti-thioredoxin (clone 2G11) staining is shown as red. Co-localization of CD44 and thioredoxin staining is shown as yellow. The magnification of the micrographs is a ×630 (B). ELISpot assay (20 h) showing the number of thioredoxin-secreting cells per 10^5 cells represented by monocytes (stimulated with 300 ng/mL PMA/300 ng/mL ionomycin), stromal cells (SC) and detergent-lyzed (Tween 20) SC. The diagram shows mean values ± SE from six replicate wells in one of four separate experiments.

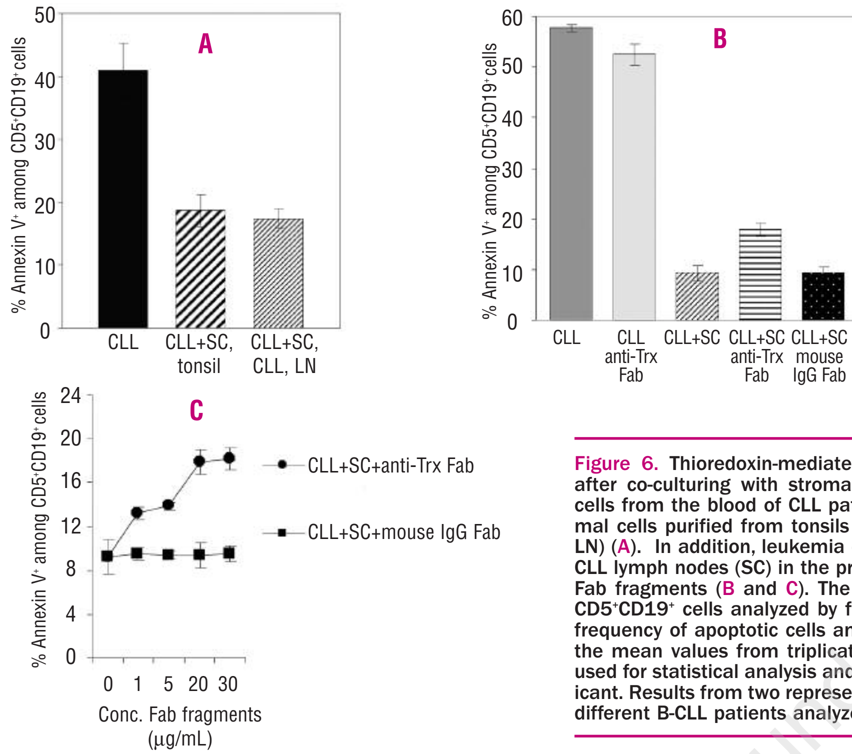

Figure 6. Thioredoxin-mediated improvement of leukemic cell survival after co-culturing with stromal cells from CLL lymph nodes. Leukemia cells from the blood of CLL patients were cultured with and without stromal cells purified from tonsils (SC, Tonsil) or CLL lymph nodes (SC, CLL, LN) (A). In addition, leukemia cells were cultured with stromal cells from CLL lymph nodes (SC) in the presence or absence of anti-thioredoxin (Trx) Fab fragments (B and C). The frequency of annexin V$^+$ cells among the CD5$^+$CD19$^+$ cells analyzed by flow cytometry was used to determine the frequency of apoptotic cells among the leukemia cells. The figures show the mean values from triplicate experiments ± SE. Student´s t-test was used for statistical analysis and p<0.05 was considered statistically significant. Results from two representative patients (ID-13 and ID-14) from five different B-CLL patients analyzed (ID-11, ID-13 to ID-16) are presented.

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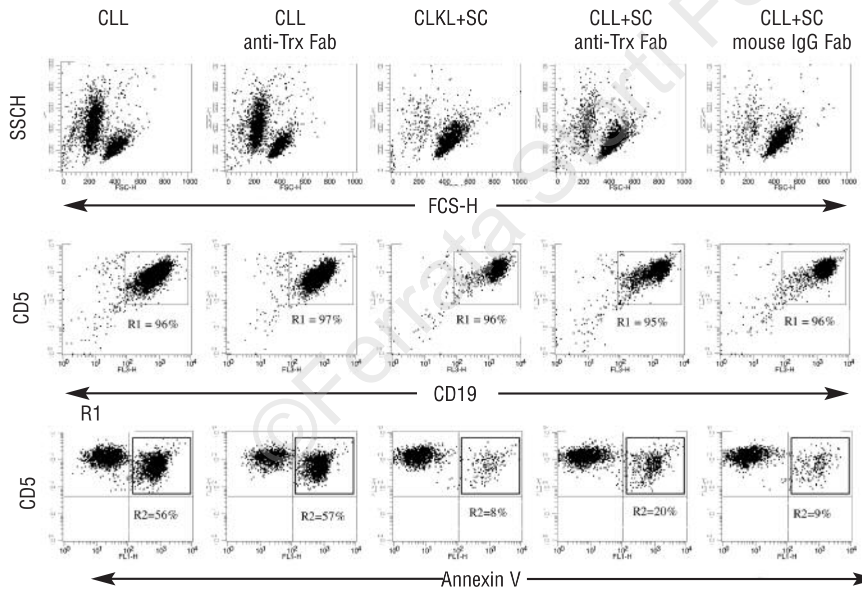

Figure 7. Percentage of annexin-V positive cells among the CD5$^+$CD19$^+$ leukemic cells after co-culturing with stromal cells in the presence or absence of anti-thioredoxin Fab fragments. After 72 h co-culturing the leukemic cells were harvested and analyzed by flow cytometry. The cells from each group (CLL, CLL$^+$anti-thioredoxin Fab, CLL+SC, CLL$^+$SC+anti-thioredoxin Fab and CLL$^+$mouse IgG Fab) were triple-stained with CD5-RPE (clone DK23), CD19-RPE-Cy5 (clone HD37) and annexin-V-FITC. In the second row the percentage of CD5$^+$CD19$^+$ cells among the harvested cells is shown. The third row shows the percentage of annexin V$^+$ cells among the CD5$^+$CD19$^+$ cells (gate R1). One representative experiment out of the ones included in Figure 6B is shown.

Discussion

In this study we have demonstrated the active release of thioredoxin from accessory cells *in vivo* and the functional effect on leukemia cell survival *in vitro*. Thioredoxin, which was previously shown to protect CLL cells from apoptosis *in vitro*,[9] was expressed in the lymph nodes of CLL patients. Interestingly, the leukemia cells *per se* expressed minimal amounts of thioredoxin. This is in accordance with previous results showing that the intracellular thioredoxin level in *ex vivo* CLL cells from peripheral blood is low[17] and increases only upon potent B-cell mitogenic stimulation. The thioredoxin production in CLL lymph nodes was, in contrast, mainly attributed to accessory stromal cells (FRC and FDC). The thioredoxin producing cells with stromal-like morphology were not restricted to CLL lymph nodes, but could also be found in sections of tonsils. T cells in the CLL microenvironment have been

shown to inhibit apoptosis of CLL cells;[6] however, although T cells were found to be potent producers of interferon-γ and interleukin-4 (*Bäckman E. et al., unpublished data*) we did not find any overexpression of thioredoxin in these cells.

Accessory cells such as stromal cells are present in proliferation centers of CLL lymphoid tissues[6,7] suggesting leukemia growth-supporting properties for these cells. Interestingly, in this study the expression of thioredoxin correlated with the presence of stromal cells as well as proliferating Ki-67+ leukemia cells. In this, as in other studies, the size and number of proliferation centers varied from patient to patient. In patients with a high proportion of proliferating cells (Ki-67+) the thioredoxin-expressing cells were mainly localized to and surrounded by Ki-67+ leukemia cells, indicating that thioredoxin is a potential tumor survival factor.

Attempts have recently been made to analyze tissue biopsies for prognostic markers.[3,18] ZAP-70 overexpression has been correlated with the absence of somatic mutations of the *IGHV* genes thus suggesting that ZAP-70 is involved in a more aggressive disease process.[18] In this study, the expression of thioredoxin was not related to the mutated or unmutated status of *IGHV* genes. However, because few mutated cases were included, this may only imply that the potential leukemia growth-promoting effect mediated by thioredoxin is independent of the *IGHV* mutational status of the CLL patient.

In addition to the high expression of thioredoxin in stromal cells of CLL lymph nodes, our results demonstrated that purified stromal cells were highly potent thioredoxin-secretors *ex vivo*. The functional significance of this finding was ascertained in experiments in which we co-cultured stromal cells with CLL cells and found a decrease in the frequency of apoptotic CLL cells. This effect was specifically blocked by Fab fragments of an anti-thioredoxin monoclonal antibody. Our present results add a novel aspect to previous findings, revealing extended survival of leukemic B cells in an *in vitro* bone marrow environment.[19,20] Nurse-like cells expanded *in vitro* from CLL blood samples have also been shown to deliver growth and survival signals via CD31/CD38 and plexin-B1/CD100 ligand interactions as well as via stromal-derived factor.[21,22] Interestingly, both stromal cells retrieved from CLL bone marrow and nurse-like cells were able to secrete thioredoxin (*Bäckman E. et al., unpublished data*), indicating that the thioredoxin secretion by lymph node stromal cells was not tissue-specific. In our study, we cannot exclude that other stromal derived factors, besides thioredoxin, contributed to the improved CLL cell survival. However, it is noteworthy that the stromal cell-mediated anti-apoptotic effect was significantly blocked by anti-thioredoxin. The detailed molecular mechanism behind the improved thioredoxin-mediated CLL survival remains to be elucidated. However, it is known that the multifunctional thioredoxin protein exerts several redox-regulatory functions and physically associates with a large number of target proteins, modulating their three-dimensional structure (and functions) by catalyzing thiol-disulfide exchange reactions.[8] Interferon-γ and CD4 are cysteine-rich membrane receptors known to be redox modulated by extracellular thioredoxin.[23,24] Intracellularly, thioredoxin binds to apoptosis signal-regulated kinase-1 (ASK-1) regulating its activity.[25] In addition, the DNA binding of the NF-κB p50 subunit is regulated by thioredoxin.[26] Thus, thioredoxin is a key protein in inducing synthesis of several cytokines, including interleukin-4, interferon-γ and tumor necrosis factor, which are known to exert survival effects on CLL.[27]

In our study, we found that accessory cells produced thioredoxin, which is in contrast with reports on other leukemias, such as adult T-cell leukemia and solid tumors, in which the primary tumor cells *per se* over expressed thioredoxin.[8,28,29] However, regardless of source, an excess of thioredoxin favored enhanced survival and, in some studies, was associated with a poor prognosis for the patients.[30,31]

Secretion of thioredoxin has previously been shown to play a role in the activation of normal lymphocytes. For example, it has been shown that thioredoxin, in synergy with certain cytokines and CD40-ligation, induced S-phase entry and mitosis in normal B cells.[32] In addition, Angelini *et al.* have shown that T cells are dependent on the secretion of cysteine and thioredoxin by antigen-presenting dendritic cells for their activation.[33] Thus, we cannot exclude that thioredoxin produced by stromal cells from lymph nodes have a growth-promoting effect on normal B cells as well.

In conclusion, this study demonstrated high levels of thioredoxin in accessory stromal cells, including FRC and FDC, in lymph nodes from patients with B-CLL. In addition, the expression of thioredoxin was mainly localized to and surrounded by proliferating leukemic cells. A possible functional significance of the thioredoxin expression was shown in co-culture experiments by extended thioredoxin-dependent survival of leukemic cells under the influence of stromal cells isolated from lymph nodes. Our finding, which was limited to showing thioredoxin-dependent CLL survival *in vitro*, support future *in vivo* studies focusing on the role of thioredoxin as critical for leukemia growth-promoting properties in CLL patients.

Authors' contributions

EB designed and performed the research, analyzed the data and wrote the paper; AB performed the research, analyzed the data and wrote the paper; IL and BR performed the research and analyzed the data; GT performed the research, analyzed the data and directed the clinical data; CS, RR and ML diagnosed the patients, directed the patients' material and their clinical data; AR initiated, designed and supervised the research and analyzed the data. All authors reviewed the manuscript critically for important intellectual content and approved the final version.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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