Chronic lymphocytic leukemia is a malignancy of mature B cells that strongly depend on microenvironmental factors, and their deprivation has been identified as a promising treatment approach for this incurable disease. Cytokine array screening of 247 chronic lymphocytic leukemia serum samples revealed elevated levels of tumor necrosis factor (TNF) receptor-1 which were associated with poor clinical outcome. We detected a microenvironment-induced expression of TNF receptor-1 in chronic lymphocytic leukemia cells in vitro, and an aberrantly high expression of this receptor in the proliferation centers of patients' lymph nodes. Stimulation of TNF receptor-1 with TNF-α enhanced nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) activity and viability of chronic lymphocytic leukemia cells, which was inhibited by wogonin. The therapeutic effects of wogonin were analyzed in mice after adoptive transfer of Em-T-cell leukemia 1 (TCL1) leukemic cells. Wogonin treatment prevented leukemia development when given early after transplantation. The treatment of full-blown leukemia resulted in the loss of the TNF receptor-1 on chronic lymphocytic leukemia cells and their mobilization to blood. Targeting TNF receptor-1 signaling is therefore proposed for the treatment of chronic lymphocytic leukemia.

Correspondence:
m.seiffert@dkfz.de

Received: July 31, 2017.
Accepted: January 11, 2018.
Pre-published: January 11, 2018.

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/4/688

©2018 Ferrata Storti Foundation

Material published in Haematologica is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:
https://creativecommons.org/licenses/by-nc/4.0/legalcode.
Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:
https://creativecommons.org/licenses/by-nc/4.0/legalcode, sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.

Tumor necrosis factor receptor signaling is a driver of chronic lymphocytic leukemia that can be therapeutically targeted by the flavonoid wogonin

Claudia Dürr,1 Bola S. Hanna,1 Angela Schulz,1 Fabienne Lucas,1,2 Manuela Zucknick,2,3 Axel Benner,3 Andrew Clear,2 Sibylle Ohl,1 Selcen Öztürk,1 Thorsten Zenz,2 Stephan Stilgenbauer,4 Min Li-Weber,1 Peter H. Krammer,7 John G. Gribben,2 Peter Lichter1 and Martina Seiffert1

1Division of Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany; 2Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, UK; 3Division of Biostatistics, German Cancer Research Center (DKFZ), Heidelberg, Germany; 4Oslo Center for Biostatistics and Epidemiology; Department of Biostatistics, Institute of Basic Medical Sciences, University of Oslo, Norway; 5Molecular Therapy in Haematology and Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), and Department of Medicine V, University Hospital Heidelberg, Germany; 6Internal Medicine III, University of Ulm, Germany and 7Division of Immunogenetics, German Cancer Research Center (DKFZ), Heidelberg, Germany

ABSTRACT

Chronic lymphocytic leukemia is a malignancy of mature B cells that strongly depend on microenvironmental factors, and their deprivation has been identified as a promising treatment approach for this incurable disease. Cytokine array screening of 247 chronic lymphocytic leukemia serum samples revealed elevated levels of tumor necrosis factor (TNF) receptor-1 which were associated with poor clinical outcome. We detected a microenvironment-induced expression of TNF receptor-1 in chronic lymphocytic leukemia cells in vitro, and an aberrantly high expression of this receptor in the proliferation centers of patients’ lymph nodes. Stimulation of TNF receptor-1 with TNF-α enhanced nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) activity and viability of chronic lymphocytic leukemia cells, which was inhibited by wogonin. The therapeutic effects of wogonin were analyzed in mice after adoptive transfer of Em-T-cell leukemia 1 (TCL1) leukemic cells. Wogonin treatment prevented leukemia development when given early after transplantation. The treatment of full-blown leukemia resulted in the loss of the TNF receptor-1 on chronic lymphocytic leukemia cells and their mobilization to blood. Targeting TNF receptor-1 signaling is therefore proposed for the treatment of chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy that is tightly regulated by and dependent on microenvironmental stimuli provided in lymphoid tissues.1 CLL cells in this protective niche show increased resistance to spontaneous and drug-induced apoptosis which is causative for CLL progression and relapse. In vitro studies using co-cultures of CLL and non-malignant accessory cells mirrored this dependency, and identified several CLL-relevant factors and pathways.2-4 Comparative gene expression profiling of CLL cells isolated from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) further identified enhanced B-cell receptor (BCR)-mediated signaling and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) activity in the lymphoid microenvironment compared to blood.3 In accordance with this, CLL cell migratory capability and tissue homing were shown to influence disease pathogenesis and progression.4 Data from clinical trials revealed that treatment with kinase inhibitors targeting BTK, SYK or PI3K-δ leads to transient lymphocytosis accompanied by LN shrinkage due to CLL cell mobilization to PB.2,4 This impairment of CLL cell homing to lymphoid tissues sub-
substantially contributes to the observed high efficacy of these inhibitors. 10-11 Albeit, despite their clinical success, CLL remains an incurable disease due to clonal evolution of malignant cells under treatment, followed by drug resistance and relapse. 12 The current challenge is to develop new strategies by targeting not only CLL cells, but also the microenvironment, with the goal being that of eradicating the malignant cells.

Tumor necrosis factor (TNF)-α and its receptors (TNFR) have been identified in the sera of CLL patients in increased concentrations, and high TNF-α levels are indicative for an aggressive disease, thus suggesting a role in CLL progression. 13-16 TNF-α was shown to act as an autocrine growth factor in CLL. 17-18 The inhibition of TNFR signaling by etanercept, a recombinant TNF-2 derivative, in combination with the anti-CD20 antibody rituximab, caused durable remissions in refractory patients without 17p deletion. 19 However, the detailed pathomechanism of TNFR signaling in CLL development and progression remains largely unknown. TNF-α is a pro-inflammatory cytokine that exerts its pleiotropic effects via two receptors, TNFR-1 (P55) and TNFR-2 (P75). 20 Only TNFR-1 is endowed with an intracellular death domain, and can thereby induce either caspase-mediated apoptosis or pro-survival signals via NFκB activation. 21 TNF-α-induced NFκB activation was shown to be blocked by wogonin, a naturally occurring flavonoid, resulting in a shift of TNFR-1 signaling towards apoptosis induction. 22 In a multitude of in vitro and in vivo studies, wogonin has been demonstrated to exert anti-oxidant, anti-inflammatory and anti-tumor activities. 23 To elucidate the oncogenic role of TNFR-1 in CLL and to test its potential as a therapeutic target, we analyzed TNFR-1 expression and function in primary CLL cell cultures and Eμ-T-cell leukemia 1 (TCL1) mice in the presence and absence of wogonin, and used these platforms for pre-clinical evaluation of TNFR-1 as a drug target in CLL.

Methods

Samples
PB of CLL patients (Online Supplementary Table S1) and healthy donors (HD) was obtained after informed consent and in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board.

Quantification of Soluble TNFR-1

Sera from 247 CLL patients from the German CLL8 study (Online Supplementary Table S2) and from 50 age- and sex-matched healthy controls were used to quantify soluble TNFR-1 by cytometric bead arrays (BD Biosciences, Heidelberg, Germany), according to the manufacturer’s protocol. Capture beads were synthesized by coupling anti-TNFR-1 antibody (Duoset, R&D Systems, Minneapolis, MN, USA) to functionalized beads. A biotinylated detection antibody (Duoset, R&D Systems) and a streptavidin conjugate were used for visualization. Data was acquired on a FACSCanto II flow cytometer and analyzed with BD FACSDiva software (BD Biosciences). Glass slides were scanned on an Agilent microarray scanner (Agilent Technologies, Santa Clara, CA, USA) and data was analyzed using GenePix Pro software (Molecular Devices, San José, CA, USA).

Serum soluble (s)TNFR-1 was quantified in mice after adoptive transfer (AT) of CLL using mouse sTNFR-1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

Statistical analysis
Details of statistical analysis are provided in the Online Supplementary Methods.

Gene expression analysis
CLL cells and CD19-sorted B cells of HD were cultured for one day in high cell density (1x10⁷ cells in 4 mL per well in 6-well plates) and total ribonucleic acid (RNA) was isolated before (day 0 [d0]) and after culture (d1). Microarray-based transcriptome analysis and quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described. 3

Tissue microarrays and immunohistochemistry
Tissue microarrays (TMA) including BM trephines (n=20 CLL patients; n=16 HD), LN sections (n=58 CLL patients; n=14 sex-matched controls by cytometric bead arrays. The sTNFR-1 serum level predicts overall survival and tumor-associated deaths in CLL

Animal models and treatments
AT of TCL1 splenocytes was performed as previously described. 24 Briefly, 6-8-week-old C57BL/6 WT females (Charles River Laboratories, UK) were transplanted with 4x10⁷ splenocytes pooled from leukemic TCL1 mice 24 (>95% CD19+CD5+ cells) via tail vein injection. In early treatment studies, animals were randomized to daily treatment with 40 mg/kg wogonin in H₂O containing arginine as an adjuvant to improve the solubility of wogonin, administered by intraperitoneal (i.p.) injection from 48 hours after AT for three weeks. For late treatment studies, PB tumor load was determined 21 days post-AT and animals were randomized to treatment with phosphate buffered saline (PBS) or 40 mg/kg wogonin (BIOTREND Chemicals AG, Wangen, Switzerland) solved in dimethyl sulfoxide (DMSO) for 21 days using daily oral gavage. At the endpoints, serum, peritoneal exudate, PB and single-cell suspensions of lymphoid organs were prepared as described previously. 25

Results

sTNFR-1 serum level predicts overall survival and tumor-associated deaths in CLL

sTNFR-1 was quantified in serum from 247 CLL patients (Online Supplementary Table S2) and 50 age- and sex-matched controls by cytometric bead arrays. The analysis revealed a significantly higher median serum concentration of sTNFR-1 in CLL patients (2.30 ng/mL, range: 0.50-7.31) compared to controls (1.35 ng/mL, range: 0.76-1.74; P<0.0001; Figure 1A). Further, sTNFR-1 serum concentrations moderately but significantly correlated with β₂-microglobulin (R=0.582; P<0.001; Figure 1B) and thymidine kinase (R=0.265; P<0.001; Figure 1C) serum levels, suggesting the malignant cells as the source of...
Of interest, high sTNFR-1 concentrations significantly correlated with shorter overall survival (OS) (Figure 1D) and a higher incidence of tumor-associated deaths (TAD) (Figure 1E) when evaluated in a univariable Cox hazard model. Moreover, multivariate analysis revealed that sTNFR-1 represents a prognostic marker for OS and TAD irrespective of age, IGHV mutational status, 11q deletion, 17p deletion, and rituximab treatment (Online Supplementary Table S3).

Microenvironment-induced expression of TNFR-1 in CLL but not in healthy donor B cells

Culturing CLL cells in high cell density provides survival supportive stimuli to the leukemic cells that would otherwise die by spontaneous apoptosis. To identify survival-stimulating pathways in CLL, we performed microarray-based gene expression profiling of CLL cells or HD B cells before and after one day of cultivation in high cell densities. Comparative analyses of the data obtained resulted in a list of 236 genes that were significantly different in their regulation between CLL and normal B cells (Online Supplementary Table S4 lists genes with the highest difference between CLL and HD). Among them, TNFR-1 (TNFRSF1A) appeared as one of the top upregulated transcripts in CLL (mean log fold change (FC) d1 vs. d0=2.06), but not in HD (FC=0.18). This finding was validated by quantitative RT-PCR using CD19-sorted CLL or normal B cells, confirming the induced expression of TNFR-1 in three out of four CLL samples (mean FC=10.13; SEM ± 3.27) but not in HD B cells (Figure 2A). We further detected significantly enhanced levels of membrane-bound TNFR-1 (mTNFR-1) in CLL cells by flow cytometry, with a mean relative median fluorescence intensity (MFI) of 1.37 (SEM ± 0.06) on freshly isolated CLL cells, and 7.35 (SEM ± 1.36) after one day of cultivation in high cell density (P<0.001; Figure 2B).

To further investigate a microenvironment-dependent regulation of TNFR-1 expression, we cocultured CD19-sorted CLL or healthy B cells for one day with CD14-sorted monocytes that have previously been shown to support CLL cell survival, and observed upregulated mTNFR-1 expression in CLL cells by 4.08-fold (SEM ± 0.70), while healthy B cells remained negative (P=0.001; Figure 2C). TNFR-1 expression in CLL cells further increased in co-cultures over seven days with a mean relative MFI of 7.85 (SEM ± 2.62) compared to 1.37 (SEM ± 0.01) in healthy B cells (Figure 2D). Similar results were obtained by culturing CLL cells in medium containing at least 20% human serum.

Previous work has suggested that CLL cells that have recently divided in lymphoid tissues, emigrate to PB as CXCR4dimCD5bright cells. Over time, they begin to reexpress CXCR4 and lose CD5 expression, before entering the lymphoid system again. To test whether TNFR-1 expression is higher in CLL cells that have just left the lymphoid microenvironment, we compared TNFR-1 levels on CXCR4dimCD5bright and CXCR4brightCD5dim CLL cells from fresh blood samples of four patients, but did not observe any significant differences between the two cell subsets (data not shown).

These data suggest that TNFR-1 expression is induced in...
CLL cells by microenvironmental stimuli, and the receptor is quickly shed from the cell surface when cells enter PB, which presumably leads to abnormally high TNFR-1 serum levels.

mTNFR-1 expression is restricted to B cells within proliferation centers

To verify microenvironment-regulated expression of TNFR-1 in vivo, we performed immunohistochemical (IHC) analysis of LN and BM sections of CLL patients and HD using antibodies specific for TNFR-1, CD20, Ki-67, CD3, and CD68. In so doing, we observed co-localization of TNFR-1 with CD20 (B-cell marker) and Ki-67 (proliferation marker; Figure 3A), but not with CD3 (T-cell marker) or CD68 (marker for myeloid cells), suggesting B cell-specific expression of TNFR-1. In LNs, TNFR-1 positive cells were mainly located within proliferation centers consisting of Ki-67 positive, large, round paraimmunoblasts in nodular areas (Figure 3A). The percentage of TNFR-1 positive cells significantly correlated with that of Ki-67 positive cells (R=0.59; P=0.0001; Figure 3B), and was slightly higher in CLL-derived LN sections (n=58) with 10.68% (SEM ± 1.93) compared to non-CLL samples (n=14) with 7.76% (SEM ± 3.42) TNFR-1 positive B cells (Figure 3C).

In both groups, the results were very heterogeneous, ranging from 0.1-51.33% in CLL and from 0-38.34% in non-CLL samples. Analysis of reactive LN sections (n=30) revealed a clear accumulation of TNFR-1 in germinal centers with 17.48% (SEM ± 0.135) positive B cells compared to 1.74% (SEM ± 0.26) in the mantle zone (MZ; P<0.0001; Figure 3D). In BM biopsies of CLL patients (n=20), 4.12% (SEM ± 0.65) of the CD20 positive cells co-expressed mTNFR-1, whereas significantly fewer co-expressing cells were detected in BM biopsies of HD (n=16; 2.38% SEM ± 0.46; P=0.04; Figure 3E). Altogether, these data show that mTNFR-1 is expressed by proliferating CLL cells that are localized in the LN and BM as well as by proliferating non-malignant B cells in germinal centers of reactive LN.

TNF-α induced NFκB activation and survival of CLL cells in vitro is reduced by wogonin

To study the downstream effects of TNFR-1 signaling in CLL, we cultured peripheral blood mononuclear cells (PBMC) from CLL patients (n=5) in 50% human serum for
one day, which induced TNFR-1 expression. Stimulation of these cells with TNF-α significantly enhanced NFκB activity, as quantified by p65 binding to immobilized NFκB consensus sequence oligonucleotides (relative mean chemiluminescence intensity (MLI): 19.25; SEM ± 1.72), and could be blocked by neutralizing TNF-α-specific antibody (MLI: 3.45; SEM ± 1.14; P=0.001; Figure 4A). Treatment of these cultures with the flavonoid wogonin, known to impair TNF-α-induced NFκB signaling,22 resulted in a reduction of relative MLI from 2.78 (SEM ± 0.60) to 0.98 (SEM ± 0.53; Figure 4B), indicating that wogonin abolished TNF-α-induced NFκB activity. Next, the effect of TNF-α in combination with wogonin on cell survival was examined. After the induction of TNFR-1 expression by 1 day of culture in high cell densities, CLL cells were treated with increasing concentrations of wogonin, 30 minutes prior to the administration of TNF-α. Wogonin treatment for 24 h resulted in a concentration-dependent reduction in cell viability, that was significantly stronger in the presence of TNF-α (Figure 4C). Treatment with 50 µM wogonin reduced average cell survival from 64.15% (SEM ± 1.80) to 44.72% (SEM ± 4.16; P=0.0003), which was further reduced to 37.92% (SEM ± 5.22; P=0.002) and 31.33% (SEM ± 5.31; P=0.009) in the presence of 10 and 50 ng/mL TNF-α, respectively. At 100 µM wogonin, survival was reduced to 27.39% (SEM ± 2.95) in the absence of TNF-α, and furthermore, to 22.13% (SEM ± 3.67; P=0.01) and 21.63% (SEM ± 3.31; P=0.009) in the presence of 10 and 50 ng/mL TNF-α, respectively. These results suggest that wogonin impacts on CLL cell viability in vitro by inhibiting TNF-α-mediated survival signals.

TNFR-1 expression and serum levels are mirrored in the Eμ-TCL1 mouse model of CLL

To investigate whether the Eμ-TCL1 mouse line, a well-established animal model of CLL,24 mirrors our findings of TNFR-1 in human CLL, we first analyzed the serum of mice with end-stage leukemia (n=5), and confirmed an average of 3.2-fold higher levels of sTNFR-1 as compared to control animals (n=3; data not shown). A significant

Figure 3. TNFR-1 is expressed by proliferating B cells in bone marrow and lymph nodes. Immunohistochemical staining of tissue microarrays containing lymph node (LN) sections and bone marrow (BM) trephine biopsies of CLL patients and healthy donors (HD) as well as sections of reactive LN were performed using CD20, Ki-67 and TNFR-1 specific antibodies. (A) Representative micrographs of Ki-67 and TNFR-1 staining of a CLL LN section. (B) Correlation of percentages of Ki-67 positive cells and TNFR-1 positive cells in 58 CLL samples (38 LN and 20 BM). (C) CD20 positive B cells that co-expressed TNFR-1 were quantified in LN sections of CLL patients (n=38) and HD (n=14); (D) in germinal centers (GC) and marginal zones (MZ) of reactive LN sections (n=30); (E) and BM trephine biopsies isolated from CLL patients (n=20) and HD (n=16). Lines indicate mean percentage of TNFR-1 positive cells and SEM. Unpaired t-test with Welch’s correction was applied for significance analysis (**P<0.0001; *P=0.04). CLL: chronic lymphocytic leukemia; TNFR-1: tumor necrosis factor receptor-1.
increase of sTNFR-1 serum levels was further induced in young syngeneic WT animals after AT of splenocytes from leukemic Eμ-TCL1 mice, which resulted in reliable and homogeneous development of CLL as formerly described. Mean concentrations of sTNFR-1 42 days after transplantation were 2.79 ng/mL (SEM ± 0.23) in TCL1 AT mice (n=7) and 2.04 ng/mL (SEM ± 0.23) in WT mice (n=6; P=0.04; Figure 5A). In addition, analysis of mTNFR-1 by flow cytometry in murine CD19+CD5+ CLL cells isolated from different tissue sites revealed significantly higher mTNFR-1 expression in the spleen, with a mean MFI of 3.53 (SEM ± 0.28) compared to 1.84 (SEM ± 0.28) in control mice (P<0.05). These results were confirmed by mTNFR-1 expression analysis in the spleen, PB and peritoneal cavity (PC) by flow cytometry. As depicted in Figure 5B, control mice exhibited a significantly lower mTNFR-1 expression in the spleen, with a mean MFI of 3.53 in control mice; 1.84 in control mice and 1.50 in treated mice (P=0.001; Figure 5B), suggesting that microenvironmental regulation of TNFR-1 expression in malignant cells is mirrored in the Eμ-TCL1 model.

Wogonin reduces CLL development in the TCL1 adoptive transfer model

As wogonin impaired NFκB activation and survival of CLL cells in vitro, we investigated its impact on leukemia development in the TCL1 AT model. Two days after transplantation of malignant cells, mice were randomized to daily treatment with 40 mg/kg wogonin or PBS by i.p. injection for three weeks (n=10; Figure 6A). Eight out of ten mice analyzed in this study responded to wogonin treatment with significantly lower spleen weights of an average of 0.25g (SEM ± 0.06) in treated mice as compared to 0.36g (SEM ± 0.04) in the control cohort (P=0.03; Figure 6B). To assess tumor load in all affected tissues, percentages of CD19+CD5+ cells of viable CD45+ cells were analyzed in the spleen, PB and peritoneal cavity (PC) by flow cytometry. As depicted in Figure 6C, control mice exhibited a median splenic tumor load of 25.78% (SEM ± 5.37), whereas CLL cells were almost completely absent in the eight mice responding to wogonin; the mean percentage of CLL cells in all treated animals, including the two non-responders, was 15.42% (SEM ± 7.52). Further, the CLL cell content was reduced by wogonin treatment from 31.14% (SEM ± 5.63) to 18.79% (SEM ± 5.99) in PB (Figure 6D), and from 56.31% (SEM ± 6.78) to 32.55% (SEM ± 10.67) in PC (Figure 6E). The proliferation rate of CLL cells in vivo was assessed by i.p. injection of 200 μg EdU 20 h prior to euthanization of mice, which confirmed the reduced proliferative activity of CLL cells in mice responding to wogonin treatment (Figure 6F). The data was too heterogeneous to reach significance, due to two animals that did not respond to treatment. But in the majority of mice, wogonin was able to control CLL development.

Wogonin impacts on TNFR-1 expression in vivo

The effect of wogonin was further investigated in mice with advanced disease where treatment was started 21 days after AT of CLL (Figure 7A). Mice were gavaged daily with either 40 mg/kg wogonin (n=5) or PBS (n=9) for three weeks. No significant difference in spleen weight was observed upon wogonin treatment (0.92g, SEM ± 0.13 in treated versus 1.08g, SEM ± 0.08 in control mice; Figure 7B), although there was a tendency, in the treated group, of slightly smaller spleens. Similar results were obtained for tumor load in the spleen, which was 61.10% (SEM ± 4.48) in wogonin-treated versus 68.28% (SEM ± 3.18) in control mice (Figure 7C). By contrast, the percentage of CD19+CD5+ cells in PB was significantly increased in treated mice (74.67%, SEM ± 3.82) compared to controls (58.84%, SEM ± 3.39; P=0.01; Figure 7D). This significant increase persisted when CLL cell percentages after treatment were normalized to CD19+CD5+ percentages at the start of treatment, which was three weeks after AT of CLL (P=0.04; Figure 7E). To analyze whether this effect might be linked to the interference of wogonin with TNFR-1 signaling, we analyzed mTNFR-1 expression on splenic CLL cells after treatment. Interestingly, wogonin induced an almost complete loss of mTNFR-1 expression in CLL cells (MFI 1.50 in treated versus 3.53 in control mice; P=0.02; Figure 7F). This is most likely due to receptor shedding, since
quantification of sTNFR-1 in the serum of these mice by ELISA revealed significantly higher levels of 4.22 ng/mL (SEM 0.34 ng/mL) in wogonin-treated mice compared to 2.79 ng/mL (SEM 0.23 ng/mL) in control mice ($P=0.005$; Figure 7G). This increase in sTNFR-1 levels in wogonin-treated mice is not a sign of disease progression, but rather a treatment effect, including enhanced TNFR-1 shedding and mobilization of leukemic cells from the spleen to PB. Despite the fact that wogonin failed to effectively control advanced disease in mice, it had an impact on TNFR-1 expression, and was associated with an increased accumulation of malignant cells in PB, suggesting that prolonged treatment with wogonin, or combinations with other drugs, might lead to CLL-effective responses.

Figure 5. CLL-associated TNFR-1 expression in $E_{μ}$TCL1 mice. (A) Syngeneic, immunocompetent C57BL/6 mice were injected (i.v.) with 4 x 10^7 splenocytes from fully leukemic $E_{μ}$TCL1 mice (TCL1 AT; $n=7$). Non-transplanted C57BL/6 mice (WT; $n=6$) were used as controls. Serum concentrations of sTNFR-1 were quantified 42 days after CLL cell engraftment via ELISA. Lines indicate mean concentrations and SEM. Unpaired Student’s $t$-test was applied for significance analysis (*$P=0.04$). (B) Single cell suspensions of peripheral blood (PB) and spleen (SP) of TCL1 AT mice ($n=9$) 42 days after transplantation were analyzed for mTNFR-1 expression by flow cytometry by gating on CD45^+CD5^+CD19^+ CLL cells. Staining of relative MFI values normalized to isotype control are depicted. Values for PB (dots) and spleen (squares) samples of each mouse (1-9) are connected by dotted lines. Paired Student’s $t$-test was applied for significance analysis ($P<0.0001$). MFI: median fluorescence intensity; IgG: immunoglobulin G; sTNFR-1: soluble tumor necrosis factor receptor-1; mTNFR-1: membrane-bound TNFR-1.

Figure 6. Wogonin reduces leukemia development in the TCL1 adoptive transfer model. (A) Syngeneic, immunocompetent C57BL/6 mice were injected (i.v.) with 4 x 10^7 splenocytes from fully leukemic $E_{μ}$TCL1 mice. Two days after transplantation, mice were either treated with 40 mg/kg/d wogonin ($n=10$) or PBS (ctl; $n=10$) by i.p. injections. Mice were sacrificed after 21 days of treatment. Serum and organs were collected. (B) Spleen weight of wogonin-treated and control mice was assessed after 21 days of treatment. (C-E) Single cell suspensions were collected from the spleen (SPL), blood (PB) and peritoneal cavity (PC). Tumor load was assessed by flow cytometry staining and is indicated as CD5^+CD19^+ cells out of CD45^+ cells in (C) SPL, (D) PB, (E) and PC. (F) Mice were injected i.p. with 0.1 mg/g EdU 20 h before sacrificing, and EdU incorporation in spleen CD5^+CD19^+ malignant cells was analyzed after Click-iT reaction by flow cytometry. Lines in all graphs indicate mean values and SEM. Paired Student’s $t$-test was applied for significance analysis (*$P<0.03$). d: day; g: gram; ctl: control.
Discussion

The relevance of microenvironmental interactions that mediate pro-survival signaling in CLL is now generally accepted. This is, however, thus far mainly based on in vitro studies, and their role in vivo still remains ill-defined. Interfering with the crosstalk of CLL cells and their microenvironment and thereby depriving malignant cells from supportive factors has become an attractive novel approach for treatment.

In the study herein, we identified TNFR-1 as a pivotal player in CLL pathology. We observed significantly elevated sTNFR-1 serum levels in CLL patients, in line with results from Digel et al.\textsuperscript{16} We further showed that sTNFR-1 levels correlate with β₂-microglobulin and thymidine kinase serum levels, which are indicative for tumor load as well as with OS and TAD of CLL patients. Thereby, the prognostic power of sTNFR-1 was independent of established prognostic markers. These findings are in line with observed correlations of serum sTNFR-1 and disease aggressiveness in CLL, breast, colon, and pancreatic cancer.\textsuperscript{25,16,29} We further showed that CLL development in Eμ-TCL1 mice is associated with elevated TNFR-1 serum levels. Taken together, our findings determine sTNFR-1 as a predictor for disease progression in CLL.

Albeit conflicting data has been presented concerning the expression of mTNFR-1 on freshly isolated PB-derived CLL cells\textsuperscript{15,30,31} our results clearly demonstrate that these TNF receptor signaling as therapeutic target in CLL

Figure 7. Wogonin impacts on TNFR-1 expression in vivo. (A) Adoptive transfer of TCL1 splenocytes was performed as described in Figure 6. At d21 after transplantation, mice were treated either with 40 mg/kg/d wogonin (n=5) or PBS (ctr; n = 9) by daily oral gavage. Mice were sacrificed after 21 days of treatment. Serum and organs were collected. (B) Spleen weight of wogonin-treated and control mice was assessed after 21 days of treatment (d42). (C–E) Single cell suspensions were collected from spleen (SPL) and blood (PB; d42). Tumor load was assessed by flow cytometry staining and is indicated as CD5⁺CD19⁺ cells out of CD45⁺ cells in (C) SPL and (D) PB (*P<0.01). (E) Percentage tumor load in blood was further normalized to percentage at start of treatment in each mouse and is presented as ratio of d42 over d21 (*P=0.04). (F) TNFR-1 expression was quantified on CD5⁺CD19⁺ cells via flow cytometry relative to isotype control antibody (mTNFR-1; *P<0.02). (G) Serum sTNFR-1 concentration was assessed by ELISA (**P=0.005). Lines in all graphs indicate mean values and SEM. Paired Student’s t-test was applied for significance analysis. d: day; g: gram; ctl: control; MFI: median fluorescence intensity; IgG: immunoglobulin G; sTNFR-1: soluble tumor necrosis factor receptor-1; mTNFR-1: membrane-bound TNFR-1.
are negative for mTNFR-1, but upregulate their expression upon in vitro cultivation. Consistent with our data, TNFR-1 expression was reported on malignant cells in diffuse large B-cell lymphoma (DLBCL), which correlated with significantly shorter OS and progression-free survival rates compared to patients with TNFR-1 negative lymphoma cells.32 TNFR-1 induction upon malignant transformation has also been described in colorectal adenoma and prostate cancer, underlining its role in carcinogenesis.33,34

BCR and TNF signaling as well as canonical NFκB activity characterize the LN microenvironment in CLL.35 TNF-1 is a pleiotropic receptor which induces either cellular activation via NFκB or apoptosis via activation of caspases. NFκB activation appears to be the default pathway resulting in expression of anti-apoptotic proteins, whereas specific inhibition of NFκB prior to TNF-α stimulation triggers cell death.36 Our data, procured from tissue microarray staining of human BM and LN sections, suggests that TNFR-1 signaling contributes to NFκB activity in CLL cells. Thereby TNFR-1 expression was enriched within B-cell rich proliferation centers in CLL samples and genetic centers in reactive LN, the sites of NFκB activity.36 Along the same line, malignant B cells in the blood of Eµ-TCL1 mice were negative for TNFR-1, but upregulated the receptor upon recirculation to the spleen, stressing the hypothesis that TNFR-1 might be involved in CLL cell activation and survival maintenance.

Inflammatory pathways are central for CLL cell survival.1 In agreement with this fact, elevated TNF-α levels were identified in CLL patients and correlated with poor prognosis.13-15 Nonetheless, data on the role of TNF-α in CLL pathogenesis are controversial. It has been suggested that it acts as an autocrine and paracrine growth factor which induces CLL cell proliferation in vitro.7,12,16 However, Foa et al demonstrated that in most of CLL cases, proliferation was reduced upon TNF-α treatment.59 In our study, TNF-α had no effect on CLL cell proliferation (data not shown), but rather induced canonical NFκB activity in CLL cells in vitro. Similar results were reported by Coscià et al., who demonstrated that NFκB is activated in CLL cells with unmaturated IGHV genes upon TNF-α exposure.60

Clinical intervention with TNF/TNF signaling in CLL is currently restricted to global TNF-α neutralization, using etanercept in combination with rituximab.19 However, less attention has been paid to the receptors that mediate the pathogenic effect of TNF-α. In particular, selective TNFR-1 inhibition showed promising results in the treatment of inflammatory diseases in mice.41-43 Wogonin, a naturally occurring monoflavonoid, was shown to attenuate TNF-α-conferred NFκB activity and thereby sensitize malignant T cells to apoptosis. Moreover, wogonin was reported to exert cytostatic and cytotoxic activities against several cancer cell lines in vitro and in vivo, accompanied by no or only mild side effects and low toxicity for non-malignant cells.44-46 The mechanism of action of wogonin is based on CDK9 inhibition and interference with reactive oxygen species (ROS) homeostasis.47,48 Wogonin was shown to shift the redox status of malignant T cells to a more reduced state by increasing H2O2 levels and decreasing -O2 levels.23 This resulted in an inhibition of the redox-sensitive protein NFκB.7,48 In the study herein, wogonin reduced TNF-α-mediated NFκB activity and induced apoptosis in CLL cells. Future studies need to investigate whether this effect is based on interference with ROS levels.

Pre-clinical testing of wogonin after adoptive transfer of CLL in mice revealed that early treatment start resulted in a reduced tumor load in all tissues affected by disease, which might be due to the inhibition of tumor cell survival or proliferation. Accordingly, studies with several cancer cell lines showed that wogonin attenuates cyclin expression.44,49,50 When we treated animals with full-blown leukemia, starting 21 days after tumor engraftment, wogonin reduced the CLL cell content in the spleen and significantly increased tumor load in PB. This effect was accompanied by the loss of mTNFR-1 expression in CLL cells and elevated sTNFR-1 serum levels, suggesting that the shedding of mTNFR-1 from the surface of CLL cells might be causally involved in the observed accumulation of cells in the blood. Targeting CLL cells with drugs that are currently used in clinical treatment for CLL is much more effective in the blood. Therefore, combination therapy approaches with wogonin and, for example, therapeutic antibodies like rituximab might result in improved treatment responses.

References

TNF receptor signaling as therapeutic target in CLL