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

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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 *Eμ*-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.[5] In accordance with this, CLL cell migratory capability and tissue homing were shown to influence disease pathogenesis and progression.[6] 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.[7-9] This impairment of CLL cell homing to lymphoid tissues sub-

stantially 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 TNFR-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 co-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 FCAP software (BD Biosciences).

Sera from final stage leukemic *Eμ*-TCL1 mice[24] presenting with splenomegaly and more than 90% leukemic cells in the blood, and matched wild-type (WT) controls were screened for 144 inflammatory factors using Mouse Cytokine Array G2000 (RayBiotech, Norcross, GA, USA), according to the manufacturer's protocol. 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 ($1x10^7$ 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 (TMAs) including BM trephines (n=20 CLL patients; n=16 HD), LN sections (n=58 CLL patients; n=14 coincidental LN taken for non-malignant pathologies with no evidence of germinal center formation), and reactive LNs (n=28) were stained with hematoxylin and eosin (H&E) and antibodies against CD20 (Dako, clone L26, Agilent), TNFR-1 (polyclonal, Abcam, Cambridge, UK), CD3 (Labvision, clone SP7, ThermoFisher), and CD68 (Dako, clone KP1, Agilent), as detailed in *Online Supplementary Methods.*

Animal models and treatments

AT of TCL1 splenocytes was performed as previously described.[25] Briefly, 6-8-week-old C57BL/6 WT females (Charles River Laboratories, UK) were transplanted with $4x10^7$ 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 H_2O 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.[26]

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 β_2-microglobulin ($R=0.582$; $P<0.001$; Figure 1B) and thymidine kinase ($R=0.263$; $P<0.001$; Figure 1C) serum levels, suggesting the malignant cells as the source of

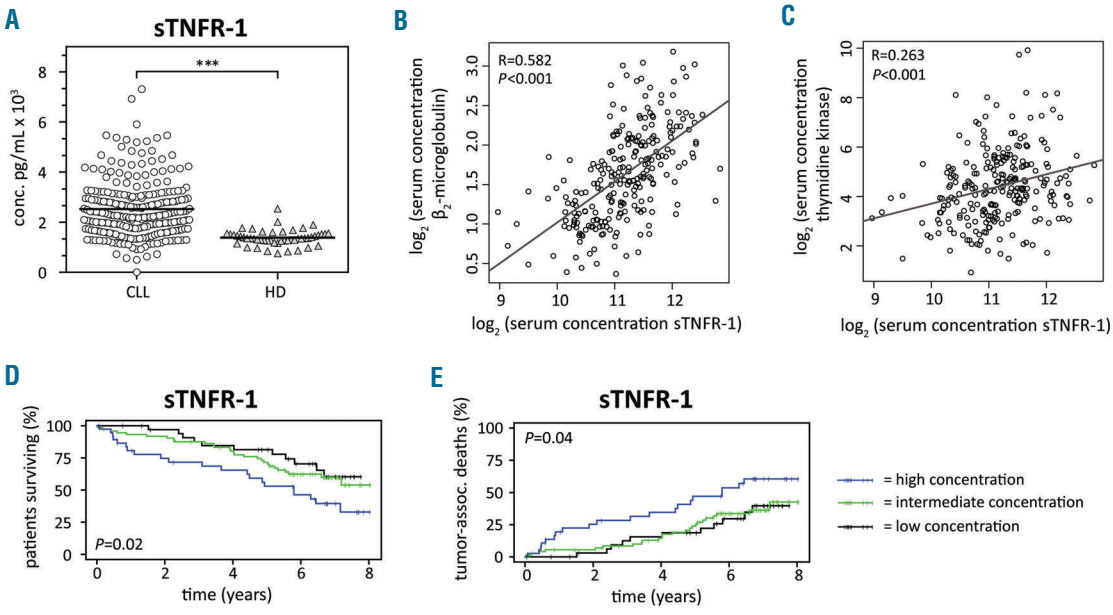

Figure 1. Elevated sTNFR-1 serum levels have prognostic relevance in CLL. (A) Serum levels of sTNFR-1 in 247 CLL patients and 50 age and sex-matched healthy donors were assessed by flow cytometry using bead arrays. The lines indicate mean concentrations. Significance analysis was performed *via* unpaired *t*-test with Welch's correction (***P<0.0001). (B) Correlations of sTNFR-1 and β_2-microglobulin, (C) as well as thymidine kinase concentrations in serum samples of 247 CLL cases are depicted. (D) Associations of $\log_2$ sTNFR-1 serum concentration and overall survival, (E) along with tumor-associated deaths of CLL patients were assessed by Cox hazard model with $\log_2$ sTNFR-1 concentration as a continuous variable. Results are depicted in a Stone-Beran estimator, with the Stone-Beran estimate at the highest concentration shown as a blue line, the Stone-Beran estimate at the median concentration shown as a green line, and the Stone-Beran estimate at the lowest concentration shown as a black line. sTNFR-1: soluble tumor necrosis factor receptor-1.

sTNFR-1. 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 $\log_2$ 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,[4] 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 $CXCR4^{dim}CD5^{bright}$ cells.[27] Over time, they begin to re-express 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 $CXCR4^{dim}CD5^{bright}$ and $CXCR4^{bright}CD5^{dim}$ 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

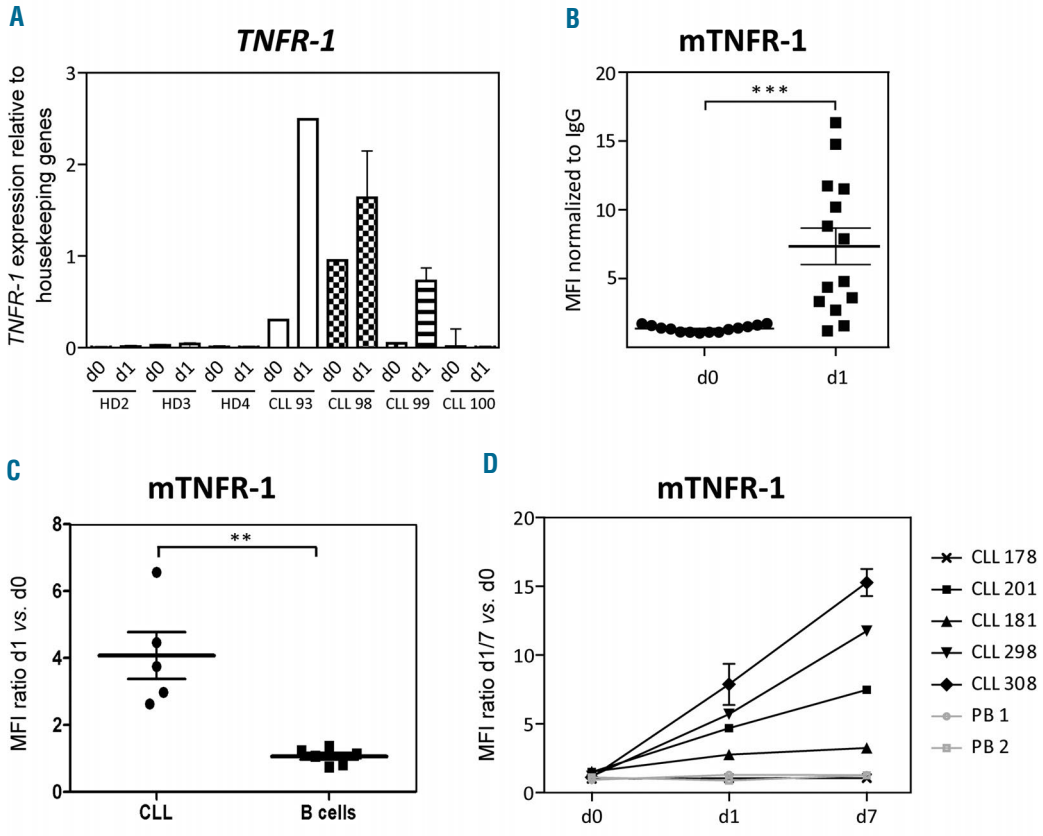

Figure 2. TNFR-1 expression in CLL cells is regulated by the microenvironment. (A) *TNFR-1* transcript levels were analyzed by quantitative RT-PCR using CD19-sorted CLL cells (n=4) or healthy donor (HD) B cells (n=3) before (day [d]0) and after 1 day (d1) of cultivation in high cell density (2.5 x 10^6 cells/mL). Results were normalized to the mean expression levels of *HPRT, DCTN2*, and *PGK*. (B) A total of 4 x 10^5 CLL peripheral blood mononuclear cells (PBMC) were cultured for 1 day in 200 µL complete medium in 96-well plates. Cell surface expression of TNFR-1 (mTNFR-1) was quantified by flow cytometry in freshly isolated cells (d0) and after 1 day of cultivation (d1; n=14). Relative median fluorescence intensity (MFI) of TNFR-1 normalized to isotype control staining was assessed by gating on $CD20^+$ lymphocytes. Lines indicate means and SEM. Significance was calculated by paired Student's *t*-test (***P=0.0005). (C+D) A total of 5 x 10^5 CD19-sorted CLL cells or healthy B cells were cocultured with 1 x 10^5 CD14-sorted monocytes for 1 or 7 days in 400 µL complete medium in 48-well plates. mTNFR-1 was quantified on $CD20^+$ lymphocytes by flow cytometry in freshly isolated cells (d0) and after cultivation (d1 and d7). Results are depicted as ratios of relative MFI on d1 or d7 *versus* d0. Lines show mean and SEM. Unpaired *t*-test with Welch's correction was applied for significance analysis (**P=0.001). CLL: chronic lymphocytic leukemia; PB: peripheral blood; mTNFR-1: membrane-bound TNFR-1.

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=0.39; 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 TNFR-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=3) in 50% human serum for

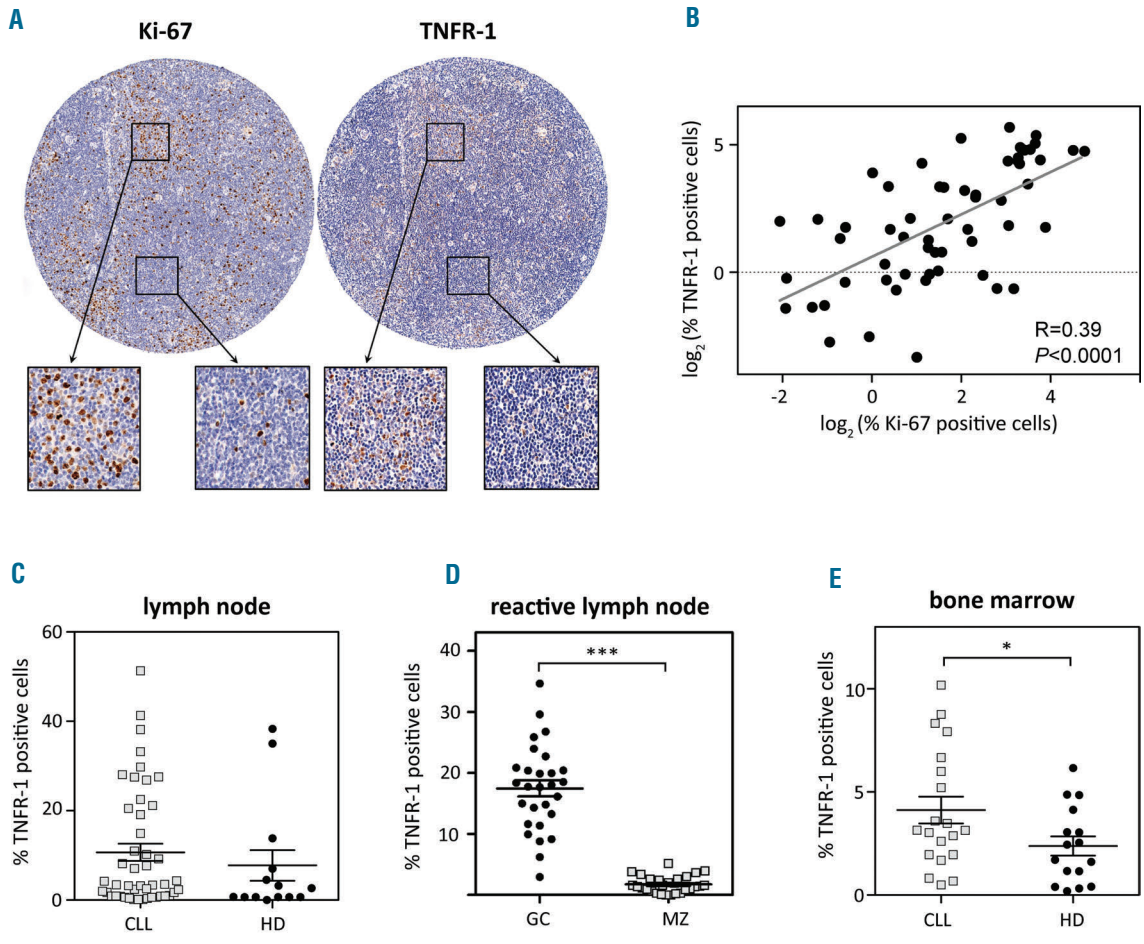

Figure 3. TNFR-1 is expressed by proliferating B cells in bone marrow and lymph nodes. Immunohistochemical staining of tissue microarrays containing lymph node (LN) sections and bone marrow (BM) trephine biopsies of CLL patients and healthy donors (HD) as well as sections of reactive LN were performed 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.

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 ± 2.99; P=0.004) 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 ± 2.67; 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

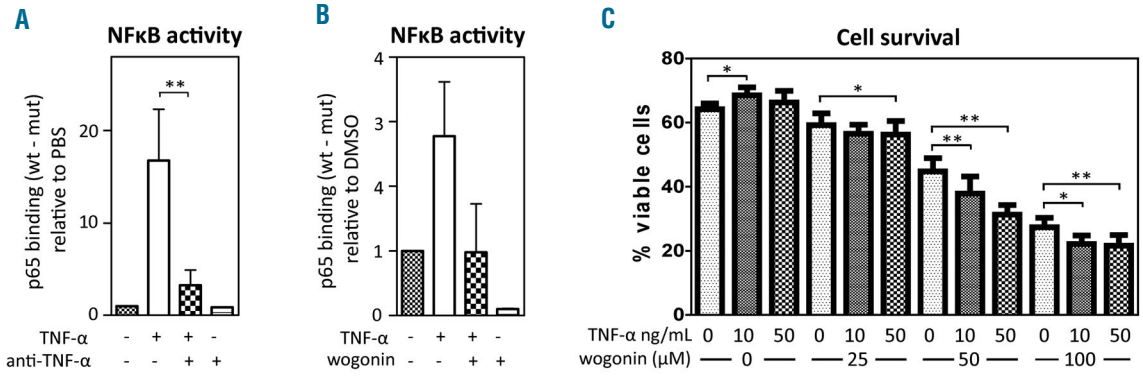

Figure 4. TNF-α-induced NFκB activity in CLL cells is inhibited by wogonin. (A) 2 x 10^7 CLL PBMC (n=3) were treated with 100 ng/mL TNF-α or PBS as control in 400 μL complete medium with or without TNF-α-blocking antibody (15 μg/mL). After 30 min, NFκB activity was assessed *via* an oligo-based chemiluminescence ELISA that detects binding of p65 to NFκB consensus binding sequence (wt), and was normalized to p65 background binding to a mutated oligo (wt – mut). Mean values and respective SEMs are depicted relative to PBS control. Paired Student's *t*-test was applied for significance analysis (***P*=0.001). (B) NFκB activity in 1 x 10^7 CLL-derived PBMC (n=2) upon addition of 100 ng/mL TNF-α for 30 minutes in the presence or absence of 50 μM wogonin was assessed as described in (A). Results are depicted as mean values and SEMs relative to DMSO control (1%). (C) A total of 4 x 10^5 CLL PBMC were cultured for 1 day in 200 μL complete medium in 96-well plates. On day 2, wogonin and TNF-α were added either alone, or in combination, at the concentrations indicated. 1% DMSO was used as untreated control. Cell survival was assessed after a further 24 h *via* flow cytometry by gating on annexin V-PE/7-AAD-negative cells. Results are depicted as mean survival rates and SEM of 5-8 samples. Paired Student's *t*-test was applied for significance analysis (**P*<0.05; ***P*<0.01). PBS: phosphate buffered saline; wt: wild-type; mut: mutated; DMSO: dimethyl sulfoxide; NFκB: nuclear factor κ-light-chain-enhancer of activated B cells; TNF-α: tumor necrosis factor-α.

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.[25,28] 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 PB (*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 23.78% (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 13.42% (SEM ± 7.32). 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/d 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.

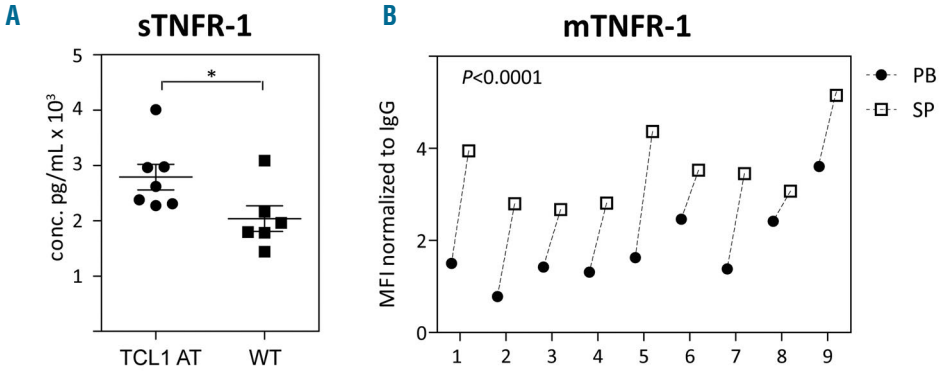

Figure 5. CLL-associated *TNFR-1* expression in *Eμ*-TCL1 mice. (A) Syngeneic, immunocompetent C57BL/6 mice were injected (i.v.) with 4 x 10^7 splenocytes from fully leukemic *Eμ*-TCL1 mice (TCL1 AT; n=7). Non-transplanted C57BL/6 mice (WT; n=6) were used as controls. Serum concentrations of sTNFR-1 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.

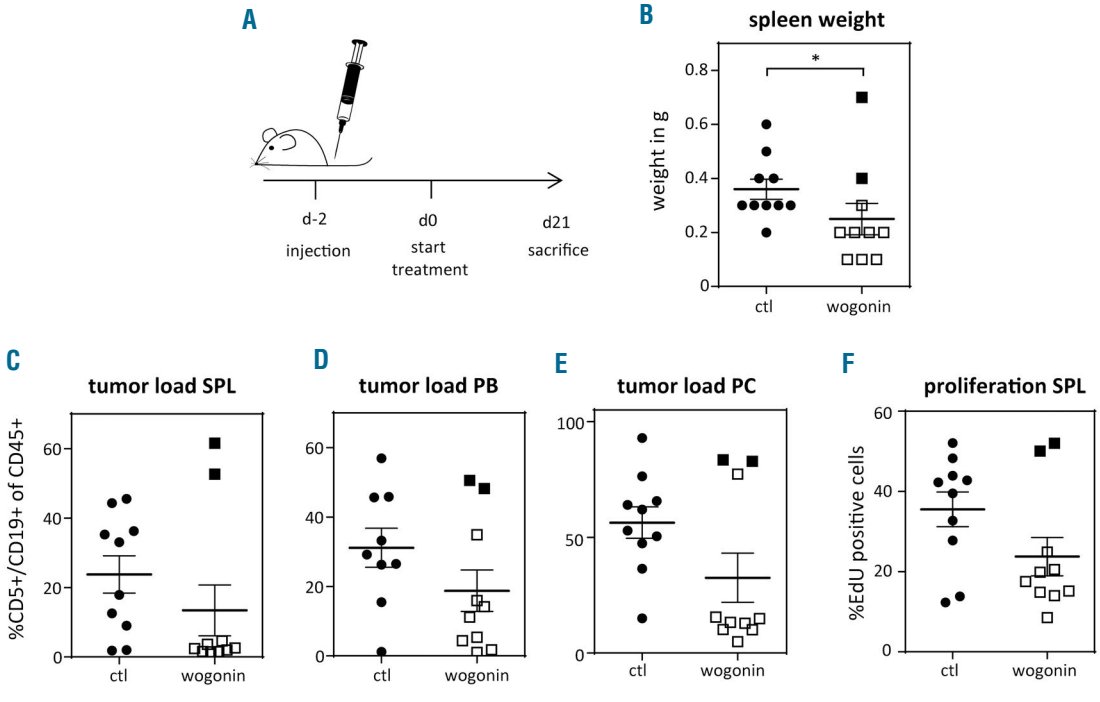

Figure 6. Wogonin reduces leukemia development in the TCL1 adoptive transfer model. (A) Syngeneic, immunocompenent C57BL/6 mice were injected (i.v.) with 4 x 10^7 splenocytes from fully leukemic *Eμ*-TCL1 mice. Two days after transplantation, mice were either treated with 40 mg/kg/d wogonin (n=10) or 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.*[16] We further showed that sTNFR-1 levels correlate with β_2-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.[15,16,29] 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,[15,30,31] our results clearly demonstrate that these

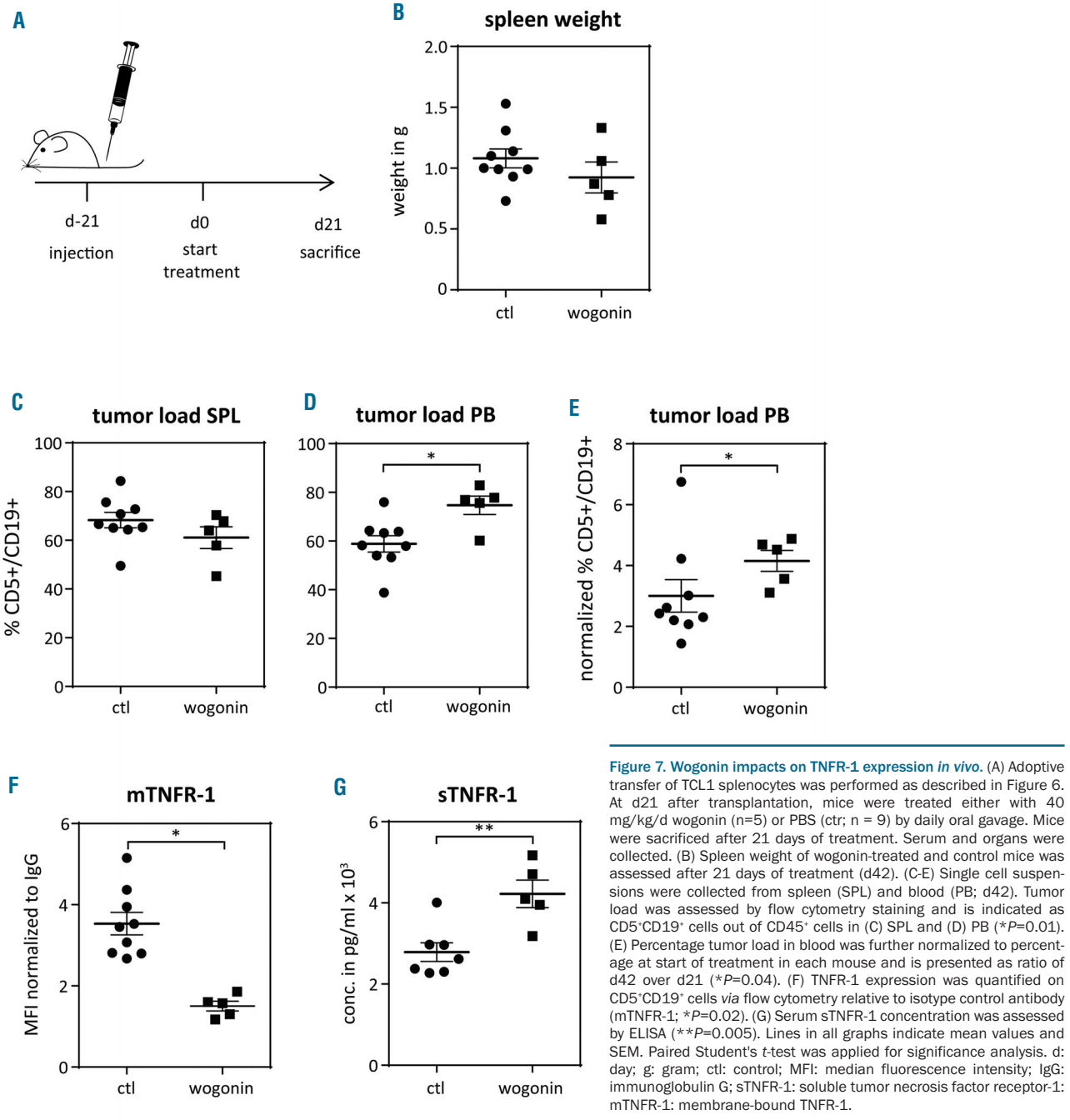

Figure 7. Wogonin impacts on TNFR-1 expression *in vivo*. (A) Adoptive transfer of TCL1 splenocytes was performed as described in Figure 6. At d21 after transplantation, mice were treated either with 40 mg/kg/d wogonin (n=5) or PBS (ctr; n = 9) by daily oral gavage. Mice were sacrificed after 21 days 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 TNFR signaling as well as canonical NFκB activity characterize the LN microenvironment in CLL.[5,35] TNFR-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 germinal centers in reactive LN, the sites of NFκB activity.[37] 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.[3] 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*.[17,18,38] However, Foa *et al*. showed that in the majority of CLL cases, proliferation was reduced upon TNF-α treatment.[39] 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 Coscia *et al*., who demonstrated that NFκB is activated in CLL cells with unmutated *IGHV* genes upon TNF-α exposure.[40]

Clinical intervention with TNF/TNFR 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.[22,45] Wogonin was shown to shift the redox status of malignant T cells to a more reduced state by increasing H_2O_2 levels and decreasing $\cdot O_2$ levels.[22] This resulted in an inhibition of the redox-sensitive protein NFκB.[47,48] 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

1. Ten Hacken E, Burger JA. Microenvironment interactions and B-cell receptor signaling in chronic lymphocytic leukemia: implications for disease pathogenesis and treatment. Biochim Biophys Acta. 2016;1863(3):401-413.
2. Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood. 1998;91(7):2387-2396.
3. Schulz A, Toedt G, Zenz T, Stilgenbauer S, Lichter P, Seiffert M. Inflammatory cytokines and signaling pathways are associated with survival of primary chronic lymphocytic leukemia cells in vitro: a dominant role of CCL2. Haematologica. 2011; 96(3):408-416.
4. Seiffert M, Schulz A, Ohl S, Dohner H, Stilgenbauer S, Lichter P. Soluble CD14 is a novel monocyte-derived survival factor for chronic lymphocytic leukemia cells, which is induced by CLL cells in vitro and present at abnormally high levels in vivo. Blood. 2010;116(20):4223-4230.
5. Herishanu Y, Perez-Galan P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood. 2011;117(2):563-574.
6. Davids MS, Burger JA. Cell trafficking in chronic lymphocytic leukemia. Open J Hematol. 2012;3(S1).
7. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. N Engl J Med. 2013;369(1):32-42.
8. Friedberg JW, Sharman J, Sweetenham J, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. Blood. 2010; 115(13):2578-2585.
9. Hoellenriegel J, Meadows SA, Sivina M, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. Blood. 2011;118(13):3603-3612.
10. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood. 2012; 119(11):2590-2594.
11. Woyach JA, Smucker K, Smith LL, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. Blood. 2014;123(12):1810-1817.
12. Woyach JA, Furman RR, Liu T-M, et al.

Resistance Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib. N Engl J Med. 2014;370(24):2286-2294.
13. Ferrajoli A, Keating MJ, Manshouri T, et al. The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia. Blood. 2002;100(4):1215-1219.
14. Adami F, Guarini A, Pini M, et al. Serum levels of tumour necrosis factor-alpha in patients with B-cell chronic lymphocytic leukaemia. Eur J Cancer. 1994;30A(9):1259-1263.
15. Bojarska-Junak A, Hus I, Szczepanek EW, Dmoszynska A, Rolinski J. Peripheral blood and bone marrow TNF and TNF receptors in early and advanced stages of B-CLL in correlation with ZAP-70 protein and CD38 antigen. Leuk Res. 2008;32(2):225-233.
16. Digel W, Porzsolt F, Schmid M, Herrmann F, Lesslauer W, Brockhaus M. High levels of circulating soluble receptors for tumor necrosis factor in hairy cell leukemia and type B chronic lymphocytic leukemia. J Clin Invest. 1992;89(5):1690-1693.
17. Digel W, Stefanic M, Schoniger W, et al. Tumor necrosis factor induces proliferation of neoplastic B cells from chronic lymphocytic leukemia. Blood. 1989;73(5):1242-1246.
18. Cordingley FT, Bianchi A, Hoffbrand AV, et al. Tumour necrosis factor as an autocrine tumour growth factor for chronic B-cell malignancies. Lancet. 1988;1(8592):969-971.
19. Woyach JA, Lin TS, Lucas MS, et al. A phase I/II study of rituximab and etanercept in patients with chronic lymphocytic leukemia and small lymphocytic lymphoma. Leukemia. 2009;23(5):912-918.
20. Tartaglia LA, Goeddel DV. Two TNF receptors. Immunol Today. 1992;13(5):151-153.
21. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell. 1996;87(3):565-576.
22. Fas SC, Baumann S, Zhu JY, et al. Wogonin sensitizes resistant malignant cells to TNFalpha- and TRAIL-induced apoptosis. Blood. 2006;108(12):3700-3706.
23. Li-Weber M. Targeting apoptosis pathways in cancer by Chinese medicine. Cancer Lett. 2013;332(2):304-312.
24. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci USA. 2002;99(10):6955-6960.
25. McClanahan F, Riches JC, Miller S, et al. Mechanisms of PD-L1/PD-1-mediated CD8 T-cell dysfunction in the context of aging-related immune defects in the Emicro-TCL1 CLL mouse model. Blood. 2015;126(2):212-221.
26. Hanna BS, McClanahan F, Yazdanparast H, et al. Depletion of CLL-associated patrolling monocytes and macrophages controls disease development and repairs immune dysfunction in vivo. Leukemia. 2016;30(3):570-579.
27. Calissano C, Damle RN, Marsilio S, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. Mol Med. 2011;17(11-12):1374-1382.
28. Hofbauer JP, Heyder C, Denk U, et al. Development of CLL in the TCL1 transgenic mouse model is associated with severe skewing of the T-cell compartment homologous to human CLL. Leukemia. 2011;25(9):1452-1458.
29. Aderka D, Englemann H, Hornik V, et al. Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients. Cancer Res. 1991;51(20):5602-5607.
30. Waage A, Liabakk N, Lien E, Lamvik J, Espevik T. p55 and p75 tumor necrosis factor receptors in patients with chronic lymphocytic leukemia. Blood. 1992;80(10):2577-2583.
31. Digel W, Schoniger W, Stefanic M, et al. Receptors for tumor necrosis factor on neoplastic B cells from chronic lymphocytic leukemia are expressed in vitro but not in vivo. Blood. 1990;76(8):1607-1613.
32. Nakayama S, Yokote T, Tsuji M, et al. TNF-alpha receptor 1 expression predicts poor prognosis of diffuse large B-cell lymphoma, not otherwise specified. Am J Surg Pathol. 2014;38(8):1138-1146.
33. Hosono K, Yamada E, Endo H, et al. Increased tumor necrosis factor receptor 1 expression in human colorectal adenomas. World J Gastroenterol. 2012;18(38):5360-5368.
34. de Miguel MP, Royuela M, Bethencourt FR, Santamaria L, Fraile B, Paniagua R. Immunoexpression of tumour necrosis factor-alpha and its receptors 1 and 2 correlates with proliferation/apoptosis equilibrium in normal, hyperplasic and carcinomatous human prostate. Cytokine. 2000;12(5):535-538.
35. Mittal AK, Chaturvedi NK, Rai KJ, et al. Chronic lymphocytic leukemia cells in a lymph node microenvironment depict molecular signature associated with an aggressive disease. Mol Med. 2014;20(1):290-301.
36. Muppidi JR, Tschopp J, Siegel RM. Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity. 2004;21(4):461-465.
37. Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol. 2000;164(4):2200-2206.
38. van Kooten C, Rensink I, Aarden L, van Oers R. Interleukin-4 inhibits both paracrine and autocrine tumor necrosis factor-alpha-induced proliferation of B chronic lymphocytic leukemia cells. Blood. 1992;80(5):1299-1306.
39. Foa R, Massaia M, Cardona S, et al. Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: a possible regulatory role of TNF in the progression of the disease. Blood. 1990;76(2):393-400.
40. Coscia M, Pantaleoni F, Riganti C, et al. IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. Leukemia. 2011;25(5):828-837.
41. Zettlitz KA, Lorenz V, Landauer K, et al. ATROSAB, a humanized antagonistic anti-tumor necrosis factor receptor one-specific antibody. MAbs. 2010;2(6):639-647.
42. Abe Y, Nomura T, Yoshioka Y, Kamada H, Tsunoda S, Tsutsumi Y. Anti-inflammatory effects of a novel TNFR1-selective antagonistic TNF mutant on established murine collagen-induced arthritis. Adv Exp Med Biol. 2011;691:493-500.
43. Shibata H, Yoshioka Y, Ohkawa A, et al. The therapeutic effect of TNFR1-selective antagonistic mutant TNF-alpha in murine hepatitis models. Cytokine. 2008;44(2):229-233.
44. Chung H, Jung Y-m, Shin D-H, et al. Anticancer effects of wogonin in both estrogen receptor-positive and -negative human breast cancer cell lines in vitro and in nude mice xenografts. Int J Cancer. 2008;122(4):816-822.
45. Polier G, Ding J, Konkimalla BV, et al. Wogonin and related natural flavones are inhibitors of CDK9 that induce apoptosis in cancer cells by transcriptional suppression of Mcl-1. Cell Death Dis. 2011;2:e182.
46. Wang W, Guo Q-L, You Q-D, et al. The anticancer activities of wogonin in murine sarcoma S180 both in vitro and in vivo. Biol Pharm Bull. 2006;29(6):1132-1137.
47. Korn SH, Wouters EF, Vos N, Janssen-Heininger YM. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. J Biol Chem. 2001;276(38):35693-35700.
48. Baumann S, Fas SC, Giaisi M, et al. Wogonin preferentially kills malignant lymphocytes and suppresses T-cell tumor growth by inducing PLCgamma1- and Ca2+-dependent apoptosis. Blood. 2008;111(4):2354-2363.
49. Yang L, Zhang HW, Hu R, et al. Wogonin induces G1 phase arrest through inhibiting Cdk4 and cyclin D1 concomitant with an elevation in p21Cip1 in human cervical carcinoma HeLa cells. Biochem Cell Biol. 2009;87(6):933-942.
50. Zhang HW, Yang Y, Zhang K, et al. Wogonin induced differentiation and G1 phase arrest of human U-937 leukemia cells via PKCdelta phosphorylation. Eur J Pharmacol. 2008;591(1-3):7-12.