The Shc family adaptor p66Shc acts as a negative regulator of proliferative and survival signals triggered by the B-cell receptor and, by enhancing the production of reactive oxygen species, promotes oxidative stress-dependent apoptosis. Additionally, p66Shc controls the expression and function of chemokine receptors that regulate lymphocyte traffic. Chronic lymphocytic leukemia cells have a p66Shc expression defect which contributes to their extended survival and correlates with poor prognosis. We analyzed the impact of p66Shc ablation on disease severity and progression in the Eμ-TCL1 mouse model of chronic lymphocytic leukemia. We showed that Eμ-TCL1/p66Shc-/-mice developed an aggressive disease that had an earlier onset, occurred at a higher incidence and led to earlier death compared to that in Eμ-TCL1 mice. Eμ-TCL1/p66Shc-/-mice displayed substantial leukemic cell accumulation in both nodal and extra-nodal sites. The target organ selectivity correlated with upregulation of chemokine receptors whose ligands are expressed therein. This also applied to chronic lymphocytic leukemia cells, where chemokine receptor expression and extent of organ infiltration were found to correlate inversely with these cells’ level of p66Shc expression. p66Shc expression declined with disease progression in Eμ-TCL1 mice and could be restored by treatment with the Bruton tyrosine kinase inhibitor ibrutinib. Our results highlight p66Shc deficiency as an important factor in the progression and severity of chronic lymphocytic leukemia and underscore p66Shc expression as a relevant therapeutic target.
regulates B-cell survival by modulating the expression of several Bcl-2 family members and inhibiting the activation of the pro-survival kinase Akt.\textsuperscript{5,6} p66\textsuperscript{Shc} also participates in B-cell trafficking by controlling CCR7, CXCR4 and S1PR1 surface expression both transcriptionally and post-translationally.\textsuperscript{24,25} Moreover, p66\textsuperscript{Shc} attenuates CXCR4 and CXCR5 signaling.\textsuperscript{5} CLL cells have a defect in the expression of p66\textsuperscript{Shc} and its transcription factor STAT4\textsuperscript{19} which is causal to their extended survival,\textsuperscript{47,71} suggesting a link between p66\textsuperscript{Shc} deficiency and the pathogenesis of CLL.

Overexpression of TCL1 driven by the IgM heavy chain enhancer (\textit{Eμ-TCL1}) in mice results in the development of a B-cell leukemia that recapitulates aggressive CLL.\textsuperscript{12} Here we have addressed the effect of p66\textsuperscript{Shc} deficiency on the onset, development and outcome of CLL by generating a \textit{Eμ-TCL1/p66\textsuperscript{Shc}-/-} mouse. We showed that p66\textsuperscript{Shc} deletion in \textit{Eμ-TCL1} mice resulted in accelerated leukemogenesis and enhanced disease aggressiveness, with enhanced nodal and extranodal infiltration. Our data provide direct evidence that p66\textsuperscript{Shc} deficiency concurs to CLL pathogenesis and highlight p66\textsuperscript{Shc} expression as a relevant disease target.

**Methods**

A detailed description of the methods is available in the Online Supplementary Data file.

**Mice**

\textit{Eμ-TCL1}\textsuperscript{12,13} and p66\textsuperscript{Shc}\textsuperscript{-/-} C57BL/6 (C57/p66\textsuperscript{-/-})\textsuperscript{14} mice were crossed to generate \textit{Eμ-TCL1/p66\textsuperscript{Shc}-/-} mice: the screening strategy is illustrated in Online Supplementary Figures S1 and S2. C57BL/6 and C57/p66\textsuperscript{-/-} mice were used as controls. Mice with ≥10% peripheral blood CD5\textsuperscript{+}CD19\textsuperscript{+} cells were considered to have developed leukemia. Overt leukemia was defined as reported elsewhere.\textsuperscript{15}

**Cell lines, patients and healthy donors**

Transfectants generated using the CLL-derived B-cell line MEC1\textsuperscript{10} and expressing human full-length p66\textsuperscript{Shc} or the p66\textsuperscript{Shc}Q mutant were described previously.\textsuperscript{26} p66\textsuperscript{Shc} silencing in an Epstein-Barr virus (EBV) B-cell line was performed as described elsewhere.\textsuperscript{11} Peripheral blood samples were collected from 157 treatment-naïve CLL patients and five CLL patients subjected to pharmacological treatments (Online Supplementary Table S1). B cells from 15 buffy coats were used as healthy population controls. B cells were purified and transfected as described previously.\textsuperscript{27}

**Immunophenotyping of leukemic cells**

Single-cell suspensions from mouse peripheral blood, peritoneal wash, spleen, bone marrow, lymph nodes, liver and lung were depleted of erythrocytes by hypotonic lysis and incubated with mouse Fc-Block for 15 min at 4°C. Murine leukemic cells and B cells purified from CLL patients and healthy donors were stained (antibodies and reagents listed in Online Supplementary Table S2) and subjected to flow cytometry (Guava Easy Cyte cytometer, Millipore).

**RNA purification and polymerase chain reactions**

Total RNA was extracted and retrotranscribed as described previously.\textsuperscript{11} Real-time polymerase chain reactions (PCR) (primers listed in Online Supplementary Table S3) were performed using GoTaq Long PCR Master Mix (Promega) and results were processed and analyzed as described elsewhere.\textsuperscript{15}

**Cell treatment, apoptosis and reactive oxygen species measurement, immunoblot and chemotaxis assays**

Freshly isolated normal and leukemic mouse B cells were treated with 50 \(\mu\text{M} \text{H}_2\text{O}_2\) for 24 h, 1 \(\mu\text{M}\) ibrutinib for 48 h, or 35 \(\mu\text{M}\) fludarabine phosphate for 16 h. Dimethylsulfoxide was used as a control. Apoptosis was measured by flow cytometry on FITC-labeled annexin-V-stained CD5\textsuperscript{+}IgM\textsuperscript{+} cells. Intracellular ROS were measured by flow cytometry in cells labeled for 30 min at 37°C with 5 \(\mu\text{M}\) CM-H\textsubscript{2}DCFDA.\textsuperscript{8}

Immunoblots and chemotaxis assays (antibodies and chemokines listed in Online Supplementary Table S2) were carried out as reported previously.\textsuperscript{11}

**Histopathology and immunohistochemistry**

Tissues, peripheral blood and peritoneal wash were collected and processed as detailed in the Online Supplementary Methods.

**Statistical analyses**

One-way analysis of variance (ANOVA) with a post-hoc Tukey test was used for experiments in which multiple groups were compared. Mann-Whitney rank-sum tests were performed to determine the significance of differences between two groups. Survival curves and medians were calculated within subgroups with the Kaplan-Meier method. A log-rank test was used to compare differences between estimated survival curves. Statistical analyses were performed using GraphPad Software (La Jolla, CA, USA). P values <0.05 were considered statistically significant.

**Study approval**

Experiments were approved by the Institutional Review Board and the local Ethics Committee.

**Results**

p66\textsuperscript{Shc} expression decreases during leukemia progression in tumoral cells from \textit{Eμ-TCL1} mice

CLL cells have a profound reduction in p66\textsuperscript{Shc} expression, which is more severe in patients with an unfavorable prognosis.\textsuperscript{4} As \textit{Eμ-TCL1} mice are a model of aggressive CLL,\textsuperscript{12} we investigated whether the p66\textsuperscript{Shc} defect in CLL cells is recapitulated in leukemic \textit{Eμ-TCL1} cells. p66\textsuperscript{Shc} mRNA was quantified in splenic leukemic cells from \textit{Eμ-TCL1} mice with overt leukemia (≥50% peripheral blood CD5\textsuperscript{+}CD19\textsuperscript{+}) in mice results in the development of mice results in the development of aggressive CLL.\textsuperscript{12} Here we have addressed the effect of p66\textsuperscript{Shc} deficiency on the onset, development and outcome of CLL by generating a \textit{Eμ-TCL1/p66\textsuperscript{Shc}-/-} mouse. We showed that p66\textsuperscript{Shc} deletion in \textit{Eμ-TCL1} mice resulted in accelerated leukemogenesis and enhanced disease aggressiveness, with enhanced nodal and extranodal infiltration. Our data provide direct evidence that p66\textsuperscript{Shc} deficiency concurs to CLL pathogenesis and highlight p66\textsuperscript{Shc} expression as a relevant disease target.
cells, p66Shc expression increased in splenic leukemic cells from Eμ-TCL1 sick mice treated with 1 μM ibrutinib for 48 h, concomitant with increased STAT4 expression (Figure 1F,G) supporting the notion that the therapeutic effects of ibrutinib are associated with its STAT4/p66Shc-elevating activity.

p66Shc deficiency accelerates leukemogenesis in Eμ-TCL1 mice

Our results suggest that the p66Shc defect observed in leukemic CLL and Eμ-TCL1 cells may be implicated in disease pathogenesis. To test this hypothesis, we transferred the p66shc-/- allele into Eμ-TCL1 mice (Online

Figure 1. p66Shc expression decreases during leukemia progression in tumoral cells from Eμ-TCL1 mice and can be restored by ibrutinib treatment. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of p66Shc mRNA in B1a, B1b, B2 and total mature B lymphocytes purified from four wildtype (WT) mice and in leukemic cells purified from five Eμ-TCL1 sick mice. The relative gene transcript abundance was determined on triplicate samples using the ΔΔCt method and normalized to GAPDH. (B, E). qRT-PCR analysis of p66Shc (B) and STAT4 (E) mRNA in B lymphocytes purified from five WT mice and in leukemic cells purified from Eμ-TCL1 mice with mild (~20% CD5+CD19+ cells in peripheral blood) (n=6) or overt leukemia (~50% CD5+CD19+ cells and white cell count >10.7 x 10^6/mL in peripheral blood) (n=5). The relative gene transcript abundance was determined on triplicate samples using the ΔΔCt method. (C) Immunoblot analysis with anti-Shc and anti-STAT4 antibodies of postnuclear supernatants of leukemic cells purified from either WT (n=3) or Eμ-TCL1 mice with mild (n=3) or overt leukemia (n=3). The stripped filters were reprobed with anti-actin antibodies. (D) Correlation between the percentages of CD5+CD19+ cells and the mRNA levels of p66Shc in peripheral blood samples obtained from Eμ-TCL1 mice at different disease stages (n=12). (F) qRT-PCR analysis of p66Shc (left) and STAT4 (right) mRNA in leukemic cells purified from spleens of Eμ-TCL1 sick mice (n=4) incubated for 48 h with either dimethylsulfoxide (DMSO) (absolute cell viability: 88.4 ± 3.2% of annexin V-/propidium iodide- cells) or 1 μM ibrutinib (absolute cell viability: 84.9 ± 2.9% of annexin V-/propidium iodide- cells). The relative gene transcript abundance was determined on triplicate samples using the ΔΔCt method. (G) Immunoblot analysis with anti-Shc and anti-STAT4 antibodies of postnuclear supernatants of leukemic cells purified from spleens of Eμ-TCL1 sick mice (n=3) incubated for 48 h with either DMSO or 1 μM ibrutinib. The stripped filters were reprobed with anti-actin antibodies. Mean ± standard deviation. One-way analysis of variance (ANOVA), multiple comparisons. ****P≤0.0001; ***P≤0.001; **P≤0.01; *P≤0.05
p66shc deletion exacerbates leukemia in TCL1 mice

Supplementary Figure S1A,B).22 As expected, Eμ-TCL1/p66Shc−/− B cells did not express p66Shc (Online Supplementary Figure S1C,D) while expressing wildtype p55, similar to Eμ-TCL1 cells (Online Supplementary Methods and Online Supplementary Table S3).22 Eμ-TCL1/p66Shc−/− mice developed a CLL-like disease similar to that occurring in Eμ-TCL1 mice. A large lymphocyte population, associated with the characteristic Gumprecht shadows, was evident in peripheral blood smears from sick mice (Online Supplementary Figure S1E). Flow cytometric analysis of peripheral blood samples from 6- and 9-month old mice revealed a discrete population of CD5+ B cells, which is the hallmark of disease development in Eμ-TCL1 mice (Online Supplementary Figures S1F and S2).22 Similar to Eμ-TCL1 mice, leukemic Eμ-TCL1/p66Shc−/− mice eventually became visibly ill, showing labored breathing and splenomegaly (Online Supplementary Figure S1G and Online Supplementary Table S4) that necessitated euthanasia.

To address the impact of p66Shc deficiency on disease onset and progression, we followed leukemia development in 85 Eμ-TCL1 and 134 Eμ-TCL1/p66Shc−/− mice by immunophenotyping monthly peripheral blood samples. Eμ-TCL1/p66Shc−/− mice showed higher white blood cell counts and higher CD5+CD19+ cell percentages in peripheral blood compared to those in Eμ-TCL1 mice (Figure 2A,B; Online Supplementary Figure S1C). Moreover disease progression, assessed as rate of increase in the percentage of CD5+CD19+ cells, was faster in Eμ-TCL1/p66Shc−/− mice (Figure 2C).

Disease incidence, defined as the percentage of sick mice (>10% peripheral blood CD5+CD19+ cells vs. ~5% in healthy C57BL/6j mice) (Online Supplementary Figure S3) at defined time points, was significantly higher in Eμ-TCL1/p66Shc−/− mice than in Eμ-TCL1 mice (Figure 2D). Moreover, p66Shc deficiency led to an earlier onset of disease, which was detected ~2 months earlier in Eμ-TCL1/p66Shc−/− mice (Figure 2D) and resulted in a shorter lifespan (Figure 2E). Hence p66Shc deficiency in Eμ-TCL1 mice accelerates disease onset and development as well as its progression to an aggressive presentation.

p66Shc deficiency enhances leukemic cell chemoresistance

Consistent with the ability of p66Shc to modulate the expression of pro- and anti-apoptotic Bcl-2 family members,17 leukemic Eμ-TCL1/p66Shc−/− cells expressed higher and lower levels of Bcl-2 and Bax, respectively, compared to levels in their Eμ-TCL1 counterparts (Figure 3A,B). At variance, Mcl-1 expression was comparable (Online Supplementary Figure S4). Accordingly, leukemic cells from sick Eμ-TCL1/p66Shc−/− mice were more resistant to fludarabine treatment, also when co-cultured with OP9 stromal cells to simulate the pro-survival stromal microenvironment (Figure 3C). Hence p66Shc deficiency in Eμ-TCL1 leukemic cells promotes these cells’ survival, which likely contributes to the faster disease development and unfavorable outcome in Eμ-TCL1/p66Shc−/− mice. This is supported by the higher sensitivity to fludarabine of leukemic cells from Eμ-TCL1 mice with milder disease (<35% peripheral blood CD5−CD19+ cells) compared to leukemic cells from mice at later disease stages (≥35% peripheral blood CD5−CD19+ cells) (Figure 3D), which may be accounted for, at least in part, by their higher residual p66Shc expression (Figure 1B,C). Consistent with this notion, tumoral Eμ-TCL1/p66Shc−/− cells were less sensitive to fludarabine treatment, independently of disease stage (Figure 3D). Hence the reduction of p66Shc expression in Eμ-TCL1 mice during disease development contributes to the decreased chemosensitivity of leukemic cells.

p66Shc deficiency in Eμ-TCL1 mice is associated with nodal and extranodal leukemic cell accumulation

Leukemic cells accumulate in the peritoneal cavity of Eμ-TCL1 mice at early stages of disease, subsequently colonizing spleen, bone marrow and lymph nodes as well as extranodal sites.22 p66Shc modulates the surface levels of receptors that regulate homing to and egress from secondary lymphoid organs,22 suggesting that its deficiency might contribute to leukemic cell accumulation in secondary lymphoid organs and infiltration into non-lymphoid organs. The extent of organ infiltration by leukemic cells was measured in spleen, bone marrow and lymph nodes from Eμ-TCL1 and Eμ-TCL1/p66Shc−/− mice with ~60% leukemic cells in peripheral blood. The analysis was extended to liver and lung, as infiltration in these organs has been documented in CLL,22 as well as to the peritoneal infiltrate.

Flow cytometric analysis of CD5−CD19+ cells revealed higher percentages of leukemic cells in lymph nodes of Eμ-TCL1/p66Shc−/− mice than in those of Eμ-TCL1 mice (Figure 4A). This was confirmed by hematoxylin & eosin staining and immunohistochemical analysis of the same organs, which highlighted massive lymph node infiltration of Eμ-TCL1/p66Shc−/− mice by small lymphoid-like B220+ cells (Figure 4A). At variance, leukemic cell accumulation in spleen and bone marrow was comparable (Online Supplementary Figure S5 and Online Supplementary Table S4), suggesting that Eμ-TCL1/p66Shc−/− tumoral cells show organ selectivity.

Leukemic cell infiltrates in the liver and lung were found to be more substantial in Eμ-TCL1/p66Shc−/− mice than in Eμ-TCL1 mice and were frequently associated with loss of organ architecture (Figure 4B,C). Increased peritoneal leukemic cell accumulation was also observed in Eμ-TCL1/p66Shc−/− mice (Figure 4D). Interestingly, flow cytometric analysis of the proliferation marker Ki-67 revealed a higher proliferation rate of leukemic Eμ-TCL1/p66Shc−/− cells in lymph nodes, liver and lung compared to their Eμ-TCL1 counterparts (Online Supplementary Figure S6), consistent with the anti-mitogenic function of p66Shc.21

Increased colonization of nodal and extranodal sites by Eμ-TCL1/p66Shc−/− cells is associated with higher expression of homing receptors

The chemokine receptors CXCR4 and CCR7 contribute to the pathogenesis of CLL by modulating leukemic B-cell homing to secondary lymphoid organs and bone marrow.22,42 p66Shc modulates surface expression of homing and egress receptors both transcriptionally and post-translationally in normal and CLL B cells,21 suggesting that the increased colonizing ability of leukemic Eμ-TCL1/p66Shc−/− cells may be caused by imbalanced expression of these receptors. Splenic leukemic cells from Eμ-TCL1 and Eμ-TCL1/p66Shc−/− mice with overt leukemia were analyzed by quantitative real-time PCR and flow cytometry for expression of chemokine receptors that regulate CLL cell homing to and residency in lymphoid organs, namely CXCR4, CCR7 and S1PR1. Splenic B cells from C57BL/J and C57BL/6/J/p66Shc−/− mice were used as controls.
Expression of CXCR4, which mainly guides B-cell homing to the bone marrow, was comparable in leukemic cells from both mouse strains (Figure 5A), accounting at least in part for the comparable extent of tumoral cell infiltration in the spleen. Conversely, surface expression of CCR7, the main lymph node B-cell homing receptor, was higher in Eμ-TCL1/p66Shc-/- cells (Figure 5B). Expression of S1PR1, which controls B-cell egress from secondary lymphoid organs, was strongly downregulated in Eμ-TCL1/p66Shc-/- compared to Eμ-TCL1 cells (Figure 5C), in agreement with the causal relationship between p66Shc and S1PR1 expression in CLL cells. Consistent with these results and the massive lymph node colonization (Figure 4A), leukemic Eμ-TCL1/p66Shc-/- cell chemotaxis towards the CCR7 ligand MIP-3β and the S1PR1 ligand S1P was enhanced and suppressed, respec-

Figure 2. p66Shc deficiency accelerates leukemogenesis in Eμ-TCL1 mice. (A, B) Flow cytometric analysis of the percentages (A) and white blood cell (WBC) counts (B) of CD5+CD19+ cells in peripheral blood samples from either Eμ-TCL1 (n=87) or Eμ-TCL1/p66Shc-/- (n=134) mice collected at the indicated months. (C) Trend-lines calculated on the monthly average percentages of CD5+CD19+ cells in the Eμ-TCL1 and Eμ-TCL1/p66Shc-/- mice shown in (A). (D) Analysis of the percentages of sick mice, calculated as the percentage of mice with ≥10% CD5+CD19+ cells, calculated on the percentages of CD5+CD19+ cells shown in (A). (E) Log-rank survival analysis of the Eμ-TCL1 or Eμ-TCL1/p66Shc-/- mice shown in (A). (D) Analysis of the percentages of sick mice, calculated as the percentage of mice with ≥10% CD5+CD19+ cells, calculated on the percentages of CD5+CD19+ cells shown in (A). (E) Log-rank survival analysis of the Eμ-TCL1 or Eμ-TCL1/p66Shc-/- mice shown in (A). Mean ± standard deviation. Mann-Whitney rank sum test. ****P≤0.0001; ***P≤0.001; **P≤0.01; ns: not significant.
tively, compared to that of leukemic Eμ-TCL1 cells, as assessed in transwell assays (Online Supplementary Figure S7). Although surface and mRNA levels of CXCR4 were similar in the two mouse strains, tumoral Eμ-TCL1/p66Shc-/- cell chemotaxis towards the CXCR4 ligand CXCL12 was enhanced (Online Supplementary Figure S7), consistent with the ability of p66Shc to negatively regulate CXCR4-dependent signaling and CXCR4 recycling in human B cells.

Lymphocyte homing to non-lymphoid organs is controlled by G protein-coupled receptors. B-cell homing to the liver is regulated by CCR1, CCR2, and CXCR3, while CCR2, CCR5, and CXCR3 have been implicated in B-cell homing to the lung.29-34 CCR1 and CCR5 mRNA levels in leukemic cells were comparable in the two mouse strains (Online Supplementary Figure S8). Conversely, both surface and mRNA levels of CCR2 and CXCR3 were higher in leukemic Eμ-TCL1/p66Shc-/- cells.
than in Eμ-TCL1 cells (Figure 5D,E). Consistent with these results and their enhanced liver and lung colonization (Figure 4B,C), chemotaxis towards the respective chemokines was enhanced in leukemic Eμ-TCL1/p66Shc−/− cells compared to leukemic Eμ-TCL1 cells (Online Supplementary Figure S7). Of note, similar effects, albeit less pronounced, were observed when mRNA and surface levels of these receptors, and the chemotactic responses thereof, were analyzed in B cells from C57BL6/J and C57BL6/J/p66Shc−/− mice (Figure 5 and Online Supplementary Figure S7), further supporting the central role of p66Shc in modulating expression of these receptors. Collectively, these results suggest that the more efficient colonization of and accumulation in extranodal sites by p66Shc−/− leukemic cells can be accounted for, at least in part, by the ability of p66Shc to modulate the expression of chemokine receptors that guide the cells’ homing to those sites.

Figure 4. Nodal and extranodal accumulation of leukemic cells lacking p66Shc. (A-D) (Left) Flow cytometric analysis of the percentages of CD5-CD19+ cells in lymph nodes (A), liver (B), lung (C) and peritoneal wash (D) from either Eμ-TCL1 (n=15) or Eμ-TCL1/p66Shc−/− (n=15) mice with overt leukemia. (Right) Hematoxylin & eosin staining (upper panels) and immunohistochemical analysis of B220 (lower panels) in lymph nodes (A), liver (B), lung (C) and peritoneal wash (D) from either Eμ-TCL1 (n=5) or Eμ-TCL1/p66Shc−/− (n=10) with overt leukemia. (Immunoperoxidase staining; original magnification, 5x, 10x and 20x). Mean ± standard deviation. Mann-Whitney rank sum test. ****P≤0.0001; ***P≤0.001; **P≤0.01.
Figure 5. p66Shc deficiency in leukemic cells results in enhanced expression of homing receptors and reduced expression of the egress receptor S1PR1. (A-E) Quantitative real-time polymerase chain reaction analysis of the mRNA levels (left) and flow cytometric analysis of surface expression (right) of CXCR4 (A), CCR7 (B), S1PR1 (C), CCR2 (D) and CXCR3 (E) in CD5+CD19+ cells purified from either wildtype (WT) (n=16) or p66Shc−/− (n=15) mice (B1a cells) and from Eμ-TCL1 (n≥16) or Eμ-TCL1/p66Shc−/− (n≥16) mice with overt leukemia. The relative gene transcript abundance was determined on triplicate samples using the ΔΔCt method. Representative flow cytometric plots of the indicated stains are shown on the right. Chemotaxis toward the respective chemokines is shown in Online Supplementary Figure S6. Mean ± standard deviation. One-way analysis of variance (ANOVA), multiple comparisons. ****P≤0.0001; ***P≤0.001; **P≤0.01; *P≤0.05.
Reconstitution of p66Shc in chronic lymphocytic leukemia cells normalizes their CCR2 and CXCR3 expression

We translated these results to human CLL cells, in which a drastic reduction in p66Shc mRNA was observed compared to levels in healthy donor B cells, with lower residual levels in patients with unmutated IGHV (UM-CLL), who develop aggressive disease,9 compared to patients with mutated IGHV (M-CLL) (Figure 6A,B). No correlation with other genetic markers of CLL, namely 13q deletion or TP53 deletion/mutation, was observed (Online Supplementary Figure S9A,B).

We investigated whether the residual levels of p66Shc in CLL cells could be correlated with the expression of the trafficking receptors found to be modulated by p66Shc deficiency in Eμ-TCL1 leukemic cells. As reported,8 surface and mRNA levels of CCR7 and S1PR1 correlated inversely and directly, respectively, with p66Shc expression in CLL cells from the patients included in this study (Figure 6C,D and Online Supplementary Figure S10A,B).
Consistent with our finding that the upregulation of surface CXCR4 in CLL cells is mainly controlled post-translationally,11 no correlation was observed between the mRNA levels of p66Shc and CXCR4 (Figure 6E and Online Supplementary Figure S10C).

The analysis was extended to CCR2 and CXCR3, which were selectively overexpressed in UM-CLL cells (Online Supplementary Figure S9C,D). Similar to CCR7, expression of these receptors was inversely correlated with that of p66Shc (Figure 6F,G and Online Supplementary Figure S10D,E), suggesting that p66Shc may negatively modulate their expression. p66Shc reconstitution in CLL cells did indeed result in a decrease in CCR2 and CXCR3 mRNA (Figure 6H and Online Supplementary Figure S10F).

Interestingly, infiltration of both nodal and extranodal areas, assessed by the number and size (cm) of infiltrated lymph nodes and the presence of spleen and/or liver enlargement, was significantly greater in patients whose leukemic cells had p66Shc mRNA levels below an arbitrarily set threshold (0.24, corresponding to the mean ΔΔCt p66Shc mRNA of all CLL patients) (Online Supplementary Figure S11 and Table 1). These data provide evidence of a correlation of the severity of the p66Shc expression defect in CLL cells with their ability to infiltrate both nodal and extranodal districts, strongly supporting a role for p66Shc deficiency in disease presentation. Of note, p66Shc expression was enhanced in CLL patients showing a significant response to second-line ibrutinib treatment but not in CLL patients that failed to respond to ibrutinib therapy (Table 1 and Online Supplementary Table S4), suggesting that the response of CLL patients to therapeutic regimens results, at least in part, from the ability of leukemic cells to restore p66Shc expression.

### Modulation of CCR2 and CXCR3 expression by p66Shc is mediated by its pro-oxidant activity

p66Shc has a ROS-elevating activity that depends on its ability to interact with cytochrome c and interrupt the respiratory chain.14 We quantified homeostatic ROS production in CLL cells loaded with the cell-permeant probe CM-H2DCFDA. ROS production was profoundly decreased in CLL B cells compared to that in normal B cells, with the lowest levels in UM-CLL patients (Figure 7A), consistent with their lowest p66Shc levels.15 Furthermore, we found a direct correlation between ROS production and p66Shc expression in CLL cells (Figure 7B). These findings were recapitulated in CM-H2DCFDA-loaded Eμ-TCL1 cells which, similar to CLL cells, express low levels of p66Shc (Figure 1B) and in which ROS production was lower than that in B cells from control C57BL/6 mice (Figure 7C), confirming the pro-oxidant activity of p66Shc.

Transcription of both ccr7 and s1pr1 is controlled in opposite directions by the ROS-elevating activity of p66Shc.16 To address the potential role of the pro-oxidant function of p66Shc in the regulation of CCR2 and CXCR3 expression we used the CLL-derived human B-cell line MEC1 stably transfected with a ROS-defective mutant carrying a E→Q substitution at positions 132-133 (p66QQ), which disrupts cytochrome c binding (Figure 7D,E).17 The empty vector transfectant lacking p66Shc (ctr) and a transfectant expressing the wildtype protein (p66) were used as controls. Flow cytometric analysis of homeostatic ROS production in the CM-H2DCFDA-loaded MEC1 transfectants showed enhanced ROS production in p66Shc-expressing cells, but not in cells expressing p66ShcQQ, compared to control cells (Figure 7F).

Mitochondrial redox signaling and apoptosis are also modulated by p53,38 which is mutated in a large proportion of CLL patients18 as well as in MEC1 cells.39 To rule out a role for *TP53* mutations in the enhanced ROS production by p66Shc-expressing MEC1 cells, ROS were measured in EBV-immortalized B cells, which express wildtype p53,38 transiently depleted of p66Shc by short interfering RNA-mediated knock-down. Similar to MEC1 cells, p66Shc deficiency in EBV-immortalized B cells resulted in a lower intracellular ROS content and enhanced CCR2 and CXCR3 expression (Online Supplementary Figure S12), underscoring the specific contribution of p66Shc to the ROS-dependent modulation of these receptors.

Surface and mRNA expression of CCR2 and CXCR3 was next measured in all transfectants. The wildtype p66Shc-expressing transfectant, but not the p66QQ transfectant, had lower mRNA and surface levels of both receptors compared to the levels in control cells (Figure 7G,I). Surface and mRNA expression of CCR2 and CXCR3 was also decreased in MEC1 cells after treatment with 50 μM H2O2, an exogenous ROS source (Figure 7H-J), indicating that the ability of p66Shc to modulate CCR2 and CXCR3 expression involves its ROS-elevating activity.

### Table 1. Pathological characteristics of patients with chronic lymphocytic leukemia and p66Shc mRNA levels in their respective leukemic cells during treatment.

| % of Patients | p66Shc mRNA (ΔΔCt) |
|---------------|-------------------|
| above threshold | ≤ 0.24 ± 0.7 |
| N. of CLL patients | 30 | 34 |
| N. of UM-CLL patients | 6 | 19 |
| % UM-CLL | 20.00 | 55.88 |
| % of infiltrated lymph nodes | 0.67 ± 0.23 | 2.27 ± 0.24 |
| N. of infiltrated lymph nodes | 0.01 below threshold | 0.001 below threshold |
| % LN > 1.5 cm | 33.33 | 88.23 |
| % Spleen infiltration (> 13 cm) | 13.33 | 88.23 |
| % Liver infiltration (> 1 cm under arch) | 3.33 | 39.14 |

**Patients responding to ibrutinib**

| % of Patients | Patients “failing” ibrutinib |
|---------------|-----------------------------|
| Before CIT | 0.8±0.2 | 0.14±0.06 |
| Follow-up CIT | 5.8±2.3 | 0.35 |
| Before ibrutinib | 1.1±0.4 | 0.12±0.08 |
| Follow-up ibrutinib | 26.1±6.9 | 0.23±0.22 |

* Patients with chronic lymphocytic leukemia (CLL) were grouped according to p66Shc mRNA expression into either “above threshold” and “below threshold” threshold 0.24, corresponding to the mean ΔΔCt p66Shc mRNA of all CLL patients. Patients with p66Shc mRNA expression above the threshold were considered to be responders, while patients with p66Shc mRNA expression below the threshold were considered to be “failing” or “non-responding” based on International Working Group CLL response criteria. Mann-Whitney rank sum test. **P<0.001; *P<0.05. LN: lymph nodes; CLI: chronic lymphocytic leukemia; UM-CLL: unmutated CLL; CIT: chemo-immunotherapy.
Figure 7. The pro-oxidant activity of p66Shc modulates CCR2 and CXCR3 expression. (A, C) Flow cytometric analysis of reactive oxygen species (ROS) production in B cells purified from either healthy donors (HD, n=7) or patients with chronic lymphocytic leukemia (CLL) grouped according to whether they had mutated CLL (M-CLL) (n=11) or unmutated CLL (UM-CLL) (n=9) (A) and in B1a cells from wildtype (C57BL/6, n=9) mice and from Eμ-TCL1 (n=13) or Eμ-TCL1/p66Shc−/− (n=12) sick mice (C), loaded with CM-H2DCFDA. Data refer to duplicate samples from each patient/donor/mouse. (B) Correlation between mRNA levels of p66Shc and ROS production in B cells purified from CLL patients (n=28). (D, E) Immunoblot analysis of Shc expression (D) and quantitative real-time polymerase chain reaction (qRT-PCR) analysis of p66Shc mRNA (E) in MEC1 B cells stably transfected with empty vector (ctr) or an expression construct encoding either wildtype p66Shc (p66) or the EE132/133QQ (p66QQ) mutant, and in B cells purified from healthy donors (HD) (n=3). A control anti-actin blot of the stripped filter is shown below. The migration of molecular mass markers is indicated. The domain structure of p66Shc showing the localization of the amino acid residues substituted in the mutants is schematized at the top of the panel. (F) Flow cytometric analysis of ROS production in the MEC1 B-cell transfectants and in B cells purified from healthy donors (B cell, n=5) loaded with CM-H2DCFDA. Data refer to duplicate samples from five independent experiments. (G, I) Flow cytometric analysis (G) and qRT-PCR analysis of the mRNA levels (I) of CCR2 (left) and CXCR3 (right) in MEC1 transfectants. Data refer to duplicate samples from five independent experiments. (H, J) Flow cytometric analysis (H) and qRT-PCR analysis of the mRNA levels (J) of CCR2 and CXCR3 in MEC1 cells treated for 24 h with either dimethylsulfoxide (DMSO) or 50 μM H2O2. Data refer to duplicate samples from four independent experiments. The relative gene transcript abundance was determined on triplicate samples using the ΔΔCt method. Mean ± standard deviation. Mann-Whitney rank sum test. ****P<0.0001; ***P<0.001**P<0.01; *P<0.05. MFI: mean fluorescence intensity.
Discussion

Here we used a genetic approach to specifically assess the outcome of p66Shc deficiency on CLL cell survival and disease onset and development. We showed that p66Shc deletion in Eμ-TCL1 mice results in accelerated leukemogenesis and enhanced disease aggressiveness, with massive nodal and extranodal infiltration. The enhanced ability of leukemic p66Shc−/− cells to infiltrate organs was associated with increased expression of chemokine receptors that drive homing to the organs. p66Shc expression declined with disease progression in Eμ-TCL1 cells, similar to human CLL. This defect could be restored by ibrutinib treatment which enhanced the cells’ chemosensitivity. These results demonstrate in vivo that the p66Shc defect found in CLL cells concurs to CLL pathogenesis. Of note, p66Shc−/− mice spontaneously develop age-related autoimmune, a feature frequently associated with CLL. Interestingly, p66Shc downregulation in CLL B cells induces the expression of the inhibitory molecule ILT3, suggesting that compensatory mechanisms might be operative to restrain CLL cell responses.

The negative impact of p66Shc deletion on disease progression and outcome in Eμ-TCL1 mice can be accounted for, at least in part, by the extended survival and chemoresistance of leukemic cells, even when co-cultured with stromal cells as a surrogate pro-survival microenvironment. The p66Shc expression defect in CLL contributes to this biological behavior. p66Shc deficiency does indeed impinge on the Bcl-2 family balance in B cells, contributing to the shift of CLL cells towards survival, which correlates with chemoresistance and poor prognosis. The ROS-elevating activity of p66Shc underlies this latter’s ability to modulate the genes, several of which are redox-sensitive.

The survival of CLL cells depends to a major extent on their ability to home to the pro-survival microenvironment of bone marrow and secondary lymphoid organs. This process is orchestrated by homing receptors responding to local chemokines and egress receptors responding to lymph and blood S1P. p66Shc is a central part of this circuitry which it affects by: (i) modulating CCR7 and S1PR1 expression in opposite directions in a ROS-dependent fashion; (ii) modulating CCR7 and CXCR4 by slowing down their endosomal recycling; and (iii) attenuating CXCR4 and CXCR5 signaling by recruiting the phosphatases SHP-1 and SHIP-1 close to the activated receptors. The p66Shc defect in CLL cells has a major impact on these processes, resulting in enhanced responses to the chemokines of the lymphoid niche and impaired response to S1P. This imbalance is expected to contribute to the lymphadenopathy and chemoresistance observed in a significant proportion of CLL patients, and indeed the levels of CCR7 are significantly higher and those of S1PR1 lower in CLL patients with clinical lymphadenopathy. We showed that the levels of p66Shc in leukemic cells are inversely related to both the number and size of infiltrated lymph nodes in CLL patients. The results obtained in Eμ-TCL1/p66Shc−/− mice, showing massive lymph node accumulation during disease progression, provide experimental evidence that p66Shc deficiency promotes the nodal leukemic cell accumulation in CLL. p66Shc deficiency also results in a striking extranodal accumulation of leukemic cells, with a preference for liver and lung, the most frequent extranodal target sites in CLL.

The ROS-related ability of p66Shc to modulate the expression and function of CCR2 and CXCR3, which drive neoplastic B-cell homing to liver and lung where the respective ligands are expressed, may account for the enhanced ability of leukemic Eμ-TCL1/p66Shc−/− cells to colonize these organs. Interestingly, CCR2 and CXCR3 are overexpressed in CLL cells (as shown in this study and reported by Trentin et al. for CXCR3). We show that p66Shc reconstitution in CLL cells reverses these abnormalities, validating in human CLL our finding that p66Shc deficiency contributes to CCR2 and CXCR3 overexpression in leukemic Eμ-TCL1/p66Shc−/− cells.

p66Shc expression declines during disease progression in Eμ-TCL1 mice, until its almost complete loss in mice with overt leukemia, paralleling the progressive decrease in fludarabine sensitivity of tumoral cells documented previously. p66Shc transcription is largely controlled in several primary and transformed cells, including T cells, by histone deacetylation and cytosine methylation in a CpG island within the promoter. Although methylation increases in Eμ-TCL1 mice during disease development, it is unlikely that methylation of the p66shc promoter caused its progressive silencing, as p66Shc expression is not epigenetically silenced in B cells. Rather, in these cells p66shc is transcriptionally regulated by STAT4, which is defective in CLL cells. Interestingly, p66Shc can be restored both in CLL cells and in leukemic Eμ-TCL1 cells (Figure 1E,F) by treatment with ibrutinib, which also promotes STAT4 expression in leukemic Eμ-TCL1 cells (Figure 1E,F). Ibrutinib modulates the expression of genes downstream of Btk in the BCR and CXCR4 pathways, which are implicated in CLL, suggesting that STAT4 and its target p66Shc may be regulated through these pathways. While this remains to be established, considering the pleiotropic role of p66Shc in B-cell survival and trafficking our finding suggests that direct or indirect STAT4 agonists that enhance the activity of residual STAT4 in CLL cells may normalize p66Shc expression and overcome chemoresistance in CLL. Our finding that interleukin-12, which activates STAT4, restores p66Shc expression in CLL cells supports this hypothesis. Collectively, our findings underscore the pathological outcome of p66Shc deficiency in CLL and highlight the chemokine receptor network as a central target of its activity.

Acknowledgments

The authors thank Carlo M. Croce for providing Eμ-TCL1 mice and Sonia Grassini for technical assistance. This work was supported by grants from AIRC (IG-20148) and ITT-Regione Toscana to CTB, AIRC (IG-19236) to DGE, AIRC (IG-15286) to GS and AIRC (IG-15397) to LT.

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