



Ferrata Storti Foundation

# p66Shc deficiency in the E $\mu$ -TCL1 mouse model of chronic lymphocytic leukemia enhances leukemogenesis by altering the chemokine receptor landscape

Laura Patrussi,<sup>1</sup> Nagaja Capitani,<sup>1,2</sup> Cristina Ulivieri,<sup>1</sup> Noemi Manganaro,<sup>1</sup> Massimo Granai,<sup>3</sup> Francesca Cattaneo,<sup>1</sup> Anna Kabanova,<sup>1</sup> Lucia Mundo,<sup>3</sup> Stefania Gobessi,<sup>4</sup> Federica Frezzato,<sup>5,6</sup> Andrea Visentin,<sup>5,6</sup> Francesca Finetti,<sup>1</sup> Pier Giuseppe Pelicci,<sup>7</sup> Mario M. D'Elia,<sup>2</sup> Livio Trentin,<sup>5,6</sup> Gianpietro Semenzato,<sup>5,6</sup> Lorenzo Leoncini,<sup>3</sup> Dimitar G. Efremov<sup>4</sup> and Cosima T. Baldari<sup>1</sup>

Haematologica 2019  
Volume 104(10):2040-2052

<sup>1</sup>Department of Life Sciences, University of Siena, Siena; <sup>2</sup>Department of Clinical and Experimental Medicine, University of Florence, Florence; <sup>3</sup>Department of Human Biotechnologies, University of Siena, Siena; <sup>4</sup>International Center for Genetic Engineering and Biotechnology, Trieste; <sup>5</sup>Venetian Institute of Molecular Medicine, Padua; <sup>6</sup>Department of Medicine, Hematology and Clinical Immunology Branch, Padua University School of Medicine, Padua and <sup>7</sup>European Institute of Oncology, Milan, Italy

## ABSTRACT

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 E $\mu$ -TCL1 mouse model of chronic lymphocytic leukemia. We showed that E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice developed an aggressive disease that had an earlier onset, occurred at a higher incidence and led to earlier death compared to that in E $\mu$ -TCL1 mice. E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice displayed substantial leukemic cell accumulation in both nodal and extranodal 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 E $\mu$ -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.

## Correspondence:

COSIMA T. BALDARI  
baldari@unisi.it

LAURA PATRUSSI  
patrussi2@unisi.it

Received: October 22, 2018.

Accepted: February 22, 2019.

Pre-published: February 28, 2019.

doi:10.3324/haematol.2018.209981

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures:  
[www.haematologica.org/content/104/10/2040](http://www.haematologica.org/content/104/10/2040)

©2019 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.



## Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of long-lived mature CD5<sup>+</sup> B cells in peripheral blood, bone marrow and secondary lymphoid organs.<sup>1</sup> Leukemic cell survival is associated with defective apoptosis<sup>2</sup> and, moreover, is promoted by stromal cell-derived chemokines during their transit through secondary lymphoid organs,<sup>3</sup> where they also undergo proliferation within pseudofollicles.<sup>1</sup> Imbalanced expression of chemokine receptors regulating homing to (CCR7, CXCR4, CXCR5) and egress from (S1PR1) secondary lymphoid organs concurs to retain CLL cells in the lymphoid niche.<sup>3</sup>

The adaptor p66Shc participates in signaling pathways linking oxidative stress to apoptosis.<sup>4</sup> p66Shc promotes the production of reactive oxygen species (ROS) by interrupting the mitochondrial respiratory chain through cytochrome c binding and oxidation, causing activation of the apoptotic cascade. Additionally, it negatively

regulates B-cell survival by modulating the expression of several Bcl-2 family members and inhibiting the activation of the pro-survival kinase Akt.<sup>5,6</sup> p66Shc also participates in B-cell trafficking by controlling CCR7, CXCR4 and S1PR1 surface expression both transcriptionally and post-translationally.<sup>7,8</sup> Moreover, p66Shc attenuates CXCR4 and CXCR5 signaling.<sup>9</sup> CLL cells have a defect in the expression of p66Shc<sup>6</sup> and its transcription factor STAT4<sup>10</sup> which is causal to their extended survival,<sup>6,7,11</sup> suggesting a link between p66Shc deficiency and the pathogenesis of CLL.

Overexpression of *TCL1* driven by the IgM heavy chain enhancer ( $\text{E}\mu\text{-TCL1}$ ) in mice results in the development of a B-cell leukemia that recapitulates aggressive CLL.<sup>12</sup> Here we have addressed the effect of p66Shc deficiency on the onset, development and outcome of CLL by generating a  $\text{E}\mu\text{-TCL1/p66Shc}^{-/-}$  mouse. We showed that p66Shc deletion in  $\text{E}\mu\text{-TCL1}$  mice resulted in accelerated leukemogenesis and enhanced disease aggressiveness, with enhanced nodal and extranodal infiltration. Our data provide direct evidence that p66Shc deficiency concurs to CLL pathogenesis and highlight p66Shc expression as a relevant disease target.

## Methods

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

### Mice

$\text{E}\mu\text{-TCL1}$ <sup>12,13</sup> and  $\text{p66Shc}^{-/-}$  C57BL/6J (C57/p66<sup>-/-</sup>)<sup>14</sup> mice were crossed to generate  $\text{E}\mu\text{-TCL1/p66Shc}^{-/-}$  mice: the screening strategy is illustrated in *Online Supplementary Figures S1* and *S2*. C57BL/6J and C57/p66<sup>-/-</sup> mice were used as controls. Mice with  $\geq 10\%$  peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> cells were considered to have developed leukemia. Overt leukemia was defined as reported elsewhere.<sup>13</sup>

### Cell lines, patients and healthy donors

Transfectants generated using the CLL-derived B-cell line MEC1<sup>15</sup> and expressing human full-length p66Shc or the p66ShcQQ mutant were described previously.<sup>8</sup> p66shc silencing in an Epstein-Barr virus (EBV) B-cell line was performed as described elsewhere.<sup>10</sup> 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.<sup>8</sup>

### 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.<sup>8</sup> 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.<sup>11</sup>

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

Freshly isolated normal and leukemic mouse B cells were treated with 50  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> 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<sup>+</sup>IgM<sup>+</sup> cells. Intracellular ROS were measured by flow cytometry in cells labeled for 30 min at 37°C with 5  $\mu\text{M}$  CM-H<sub>2</sub>DCFDA.<sup>8</sup>

Immunoblots and chemotaxis assays (antibodies and chemokines listed in *Online Supplementary Table S2*) were carried out as reported previously.<sup>16</sup>

### 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

### p66Shc expression decreases during leukemia progression in tumoral cells from $\text{E}\mu\text{-TCL1}$ mice

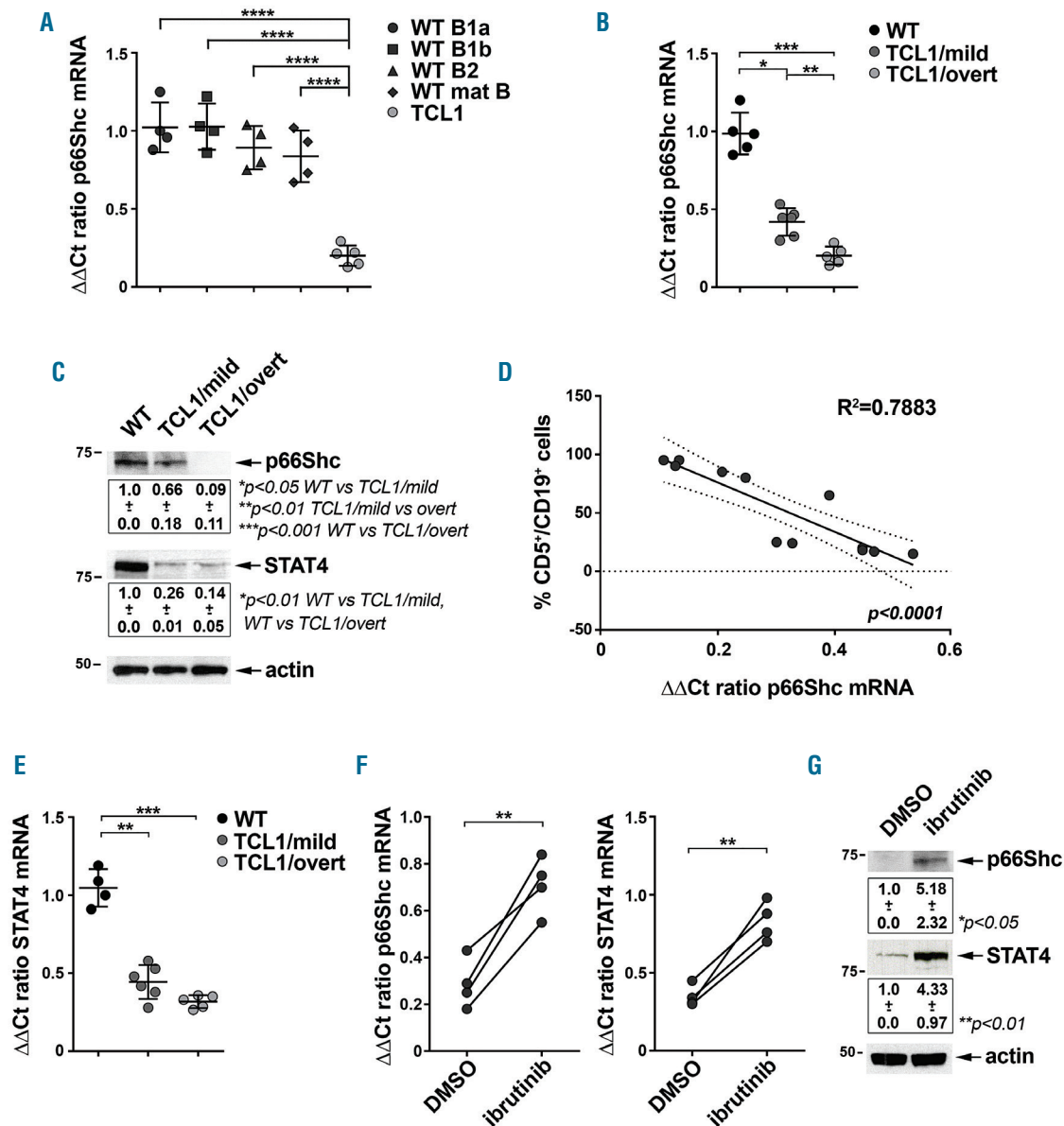
CLL cells have a profound reduction in p66Shc expression, which is more severe in patients with an unfavorable prognosis.<sup>6</sup> As  $\text{E}\mu\text{-TCL1}$  mice are a model of aggressive CLL,<sup>17</sup> we investigated whether the p66Shc defect in CLL cells is recapitulated in leukemic  $\text{E}\mu\text{-TCL1}$  cells. p66Shc mRNA was quantified in splenic leukemic cells from  $\text{E}\mu\text{-TCL1}$  mice with overt leukemia ( $\geq 50\%$  peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> cells and a white blood cell count above the normal range; see *Online Supplementary Methods*).<sup>13</sup> The analysis was extended to B1a cells from C57BL/6 mice, the normal CD5<sup>+</sup> B-cell counterpart,<sup>18</sup> and to the two other mature B-cell subsets, B1b and B2. While p66Shc was expressed at comparable levels in all normal B-cell subsets, tumoral B cells from mice with advanced disease expressed less p66Shc compared to normal B1a cells (Figure 1A). Interestingly, B1a cells from  $\text{E}\mu\text{-TCL1}$  mice with milder disease ( $\sim 20\%$  peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> cells) had intermediate levels of p66Shc (Figure 1B,C), indicating that p66Shc expression declines during disease progression, as further supported by a >78% inverse correlation between p66Shc mRNA levels in CD5<sup>+</sup>CD19<sup>+</sup> cells and the percentage of leukemic cells in peripheral blood from the same mouse (Figure 1D). STAT4, a key transcription factor for p66Shc that is defective in CLL cells,<sup>10</sup> was also downregulated in  $\text{E}\mu\text{-TCL1}$  mice (Figure 1C,E).

The p66Shc expression defect in CLL cells can be rescued by *in vitro* or *in vivo* treatment with the Btk inhibitor ibrutinib, used for CLL treatment.<sup>7,19</sup> As for human CLL

cells, p66Shc expression increased in splenic leukemic cells from E $\mu$ -TCL1 sick mice treated with 1  $\mu$ M 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 E $\mu$ -TCL1 mice

Our results suggest that the p66Shc defect observed in leukemic CLL and E $\mu$ -TCL1 cells may be implicated in disease pathogenesis. To test this hypothesis, we transferred the p66shc<sup>-/-</sup> allele into E $\mu$ -TCL1 mice (*Online*



**Figure 1.** p66Shc expression decreases during leukemia progression in tumoral cells from E $\mu$ -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 E $\mu$ -TCL1 sick mice. The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta$ Ct 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 E $\mu$ -TCL1 mice with mild ( $\sim$ 20% CD5<sup>+</sup>CD19<sup>+</sup> cells in peripheral blood) (n=6) or overt leukemia ( $\geq$ 50% CD5<sup>+</sup>CD19<sup>+</sup> cells and white cell count  $>10.7 \times 10^6$ /mL in peripheral blood) (n=5). The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta$ Ct method. (C) Immunoblot analysis with anti-Shc and anti-STAT4 antibodies of postnuclear supernatants of leukemic cells purified from either WT (n=3) or E $\mu$ -TCL1 mice with mild (n=3) or overt leukemia (n=3). The stripped filters were reprobred with anti-actin antibodies. (D) Correlation between the percentages of CD5<sup>+</sup>CD19<sup>+</sup> cells and the mRNA levels of p66Shc in peripheral blood samples obtained from E $\mu$ -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 E $\mu$ -TCL1 sick mice (n=4) incubated for 48 h with either dimethylsulfoxide (DMSO) (absolute cell viability:  $88.4 \pm 3.2\%$  of annexin V/propidium iodide cells) or 1  $\mu$ M ibrutinib (absolute cell viability:  $84.9 \pm 2.9\%$  of annexin V/propidium iodide cells). The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta$ Ct method. (G) Immunoblot analysis with anti-Shc and anti-STAT4 antibodies of postnuclear supernatants of leukemic cells purified from spleens of E $\mu$ -TCL1 sick mice (n=3) incubated for 48 h with either DMSO or 1  $\mu$ M ibrutinib. The stripped filters were reprobred with anti-actin antibodies. Mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA), multiple comparisons. \*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ ; \* $P \leq 0.05$

Supplementary Figure S1A,B).<sup>12</sup> As expected, E $\mu$ -TCL1/p66Shc<sup>-/-</sup> B cells did not express p66Shc (Online Supplementary Figure S1C,D) while expressing wildtype p53, similar to E $\mu$ -TCL1 cells (Online Supplementary Methods and Online Supplementary Table S3).<sup>20</sup> E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice developed a CLL-like disease similar to that occurring in E $\mu$ -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<sup>+</sup> B cells, which is the hallmark of disease development in E $\mu$ -TCL1 mice (Online Supplementary Figures S1F and S2).<sup>12</sup> Similar to E $\mu$ -TCL1 mice, leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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 87 E $\mu$ -TCL1 and 134 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice by immunophenotyping monthly peripheral blood samples. E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice showed higher white blood cell counts and higher CD5<sup>+</sup>CD19<sup>+</sup> cell percentages in peripheral blood compared to those in E $\mu$ -TCL1 mice (Figure 2A,B; Online Supplementary Figure S1G). Moreover disease progression, assessed as rate of increase in the percentage of CD5<sup>+</sup>CD19<sup>+</sup> cells, was faster in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice (Figure 2C).

Disease incidence, defined as the percentage of sick mice (>10% peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> cells vs. ~5% in healthy C57BL/6 mice) (Online Supplementary Figure S3) at defined time points, was significantly higher in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice than in E $\mu$ -TCL1 mice (Figure 2D). Moreover, p66Shc deficiency led to an earlier onset of disease, which was detected ~2 months earlier in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice (Figure 2D) and resulted in a shorter lifespan (Figure 2E). Hence p66Shc deficiency in E $\mu$ -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,<sup>6</sup> leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells expressed higher and lower levels of Bcl-2 and Bax, respectively, compared to levels in their E $\mu$ -TCL1 counterparts (Figure 3A,B). At variance, Mcl-1 expression was comparable (Online Supplementary Figure S4). Accordingly, leukemic cells from sick E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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 E $\mu$ -TCL1 leukemic cells promotes these cells' survival, which likely contributes to the faster disease development and unfavorable outcome in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice. This is supported by the higher sensitivity to fludarabine of leukemic cells from E $\mu$ -TCL1 mice with milder disease (<35% peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> cells) compared to leukemic cells from mice at later disease stages ( $\geq$ 35% peripheral blood CD5<sup>+</sup>CD19<sup>+</sup> 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 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells were less sensitive to fludarabine treatment, independently of disease stage (Figure 3D). Hence the reduction of p66Shc expression in E $\mu$ -TCL1 mice during disease development contributes to the decreased chemosensitivity of leukemic cells.

### p66Shc deficiency in E $\mu$ -TCL1 mice is associated with nodal and extranodal leukemic cell accumulation

Leukemic cells accumulate in the peritoneal cavity of E $\mu$ -TCL1 mice at early stages of disease, subsequently colonizing spleen, bone marrow and lymph nodes as well as extranodal sites.<sup>12</sup> p66Shc modulates the surface levels of receptors that regulate homing to and egress from secondary lymphoid organs,<sup>8,7</sup> 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 E $\mu$ -TCL1 and E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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,<sup>21,22</sup> as well as to the peritoneal infiltrate.

Flow cytometric analysis of CD5<sup>+</sup>CD19<sup>+</sup> cells revealed higher percentages of leukemic cells in lymph nodes of E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice than in those of E $\mu$ -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 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice by small lymphoid-like B220<sup>+</sup> 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 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> tumoral cells show organ selectivity.

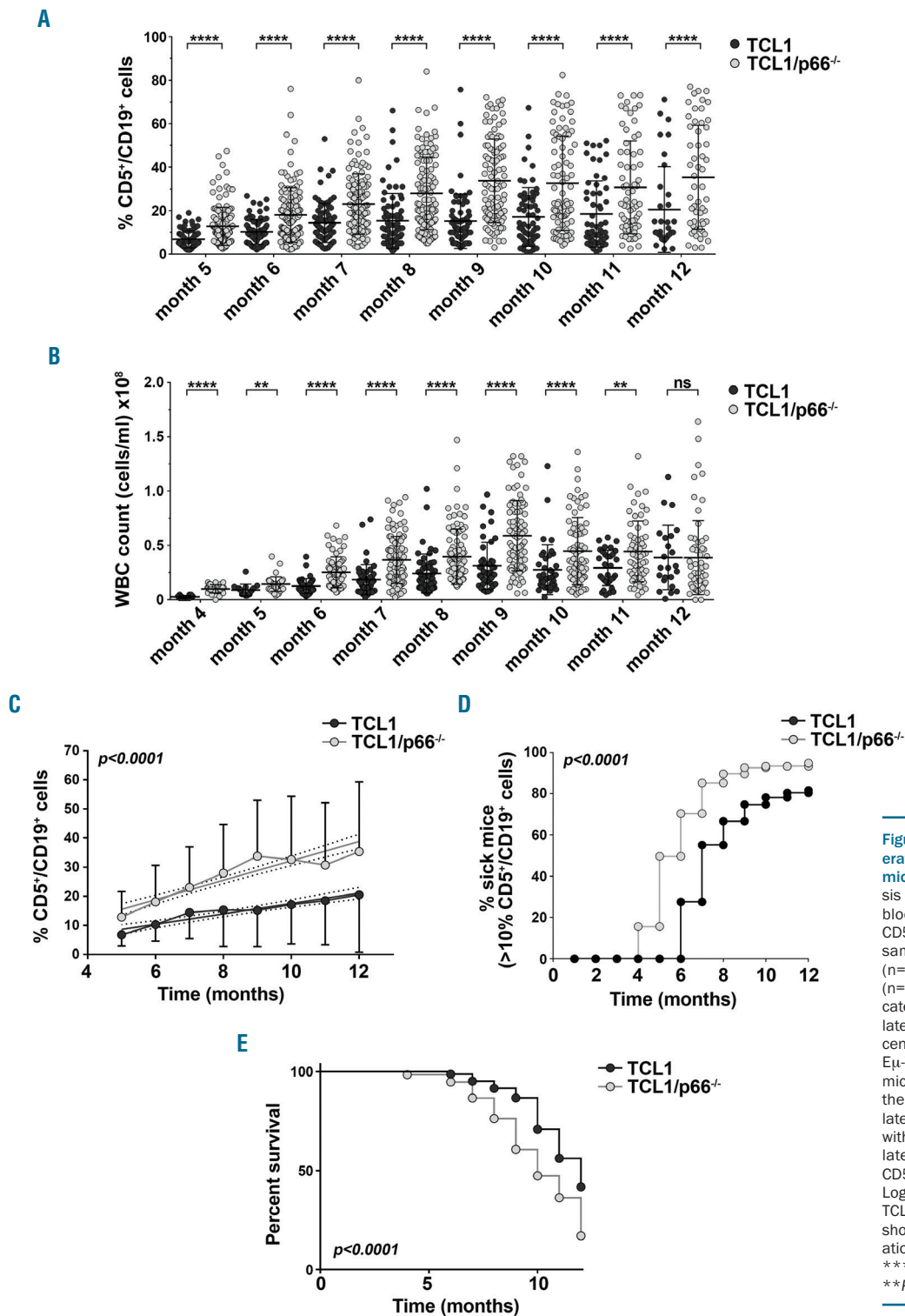
Leukemic cell infiltrates in the liver and lung were found to be more substantial in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice than in E $\mu$ -TCL1 mice and were frequently associated with loss of organ architecture (Figure 4B,C). Increased peritoneal leukemic cell accumulation was also observed in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice (Figure 4D). Interestingly, flow cytometric analysis of the proliferation marker Ki-67 revealed a higher proliferation rate of leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells in lymph nodes, liver and lung compared to their E $\mu$ -TCL1 counterparts (Online Supplementary Figure S6), consistent with the anti-mitogenic function of p66Shc.<sup>23</sup>

### Increased colonization of nodal and extranodal sites by E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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.<sup>3,24,25</sup> p66Shc modulates surface expression of homing and egress receptors both transcriptionally and post-translationally in normal and CLL B cells,<sup>7,9</sup> suggesting that the increased colonizing ability of leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells may be caused by imbalanced expression of these receptors. Splenic leukemic cells from E $\mu$ -TCL1 and E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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/6J and C57BL/6J/p66Shc<sup>-/-</sup> mice were used as controls.

Expression of CXCR4, which mainly guides B-cell homing to the bone marrow,<sup>26</sup> 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,<sup>27</sup> was higher in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells (Figure 5B). Expression of S1PR1, which controls B-cell egress from

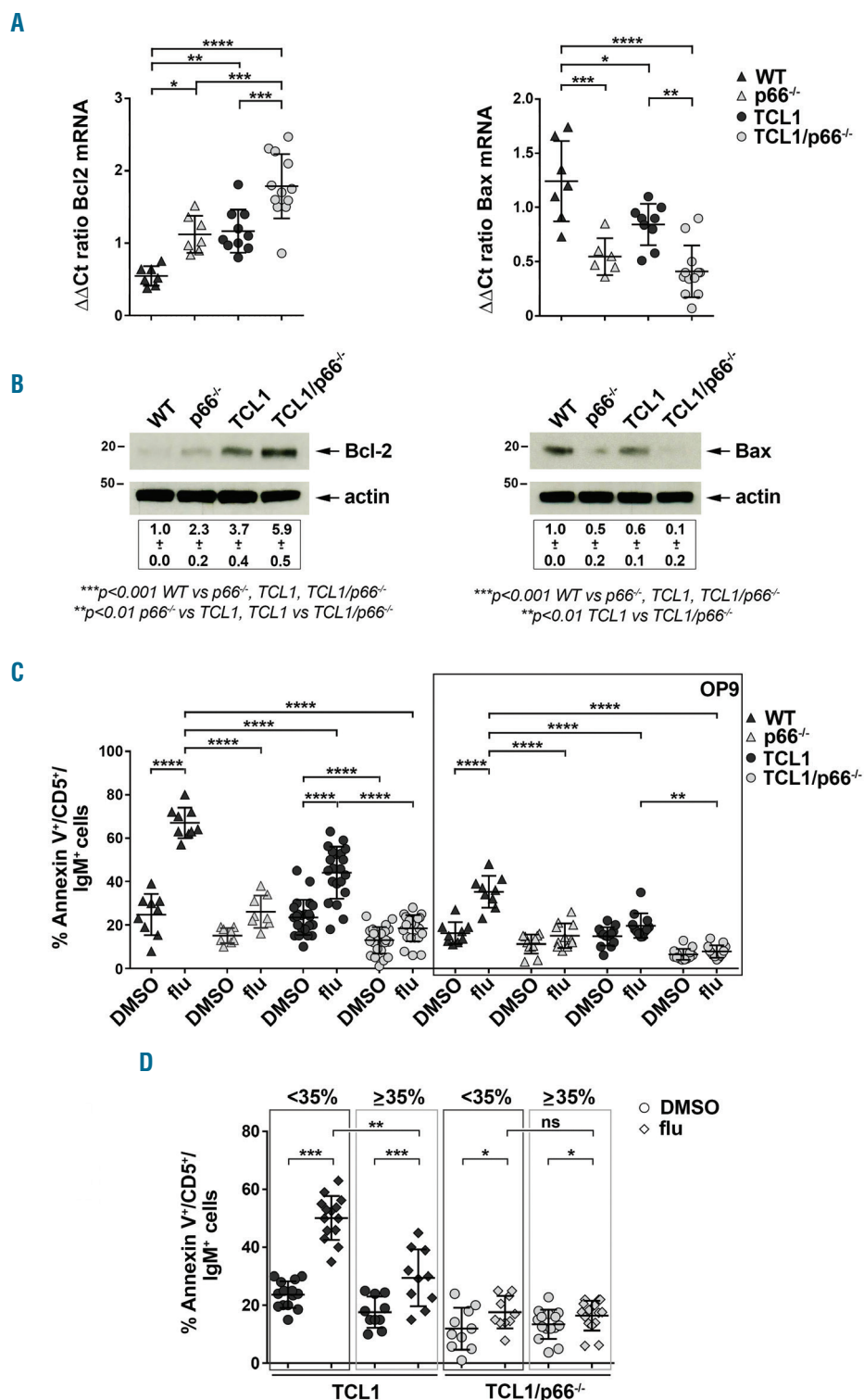
secondary lymphoid organs,<sup>28</sup> was strongly downregulated in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> compared to E $\mu$ -TCL1 cells (Figure 5C), in agreement with the causal relationship between p66Shc and S1PR1 expression in CLL cells.<sup>8</sup> Consistent with these results and the massive lymph node colonization (Figure 4A), leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cell chemotaxis towards the CCR7 ligand MIP-3 $\beta$  and the S1PR1 ligand S1P was enhanced and suppressed, respec-



**Figure 2. p66Shc deficiency accelerates leukemogenesis in E $\mu$ -TCL1 mice.** (A, B) Flow cytometric analysis of the percentages (A) and white blood cell (WBC) counts (B) of CD5<sup>+</sup>CD19<sup>+</sup> cells in peripheral blood samples from either E $\mu$ -TCL1 (n=87) or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> (n=134) mice collected at the indicated months. (C) Trend-lines calculated on the monthly average percentages of CD5<sup>+</sup>CD19<sup>+</sup> cells in the E $\mu$ -TCL1 and E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice shown in (A). (D) Analysis of the percentages of sick mice, calculated as the percentage of mice with  $\geq 10\%$  CD5<sup>+</sup>CD19<sup>+</sup> cells, calculated on the percentages of CD5<sup>+</sup>CD19<sup>+</sup> cells shown in (A). (E) Log-rank survival analysis of the E $\mu$ -TCL1 or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> mice shown in (A). Mean  $\pm$  standard deviation. Mann-Whitney rank sum test. \*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ ; 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<sup>-/-</sup> 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<sup>9</sup> and CXCR4 recycling<sup>7</sup> in human B cells.

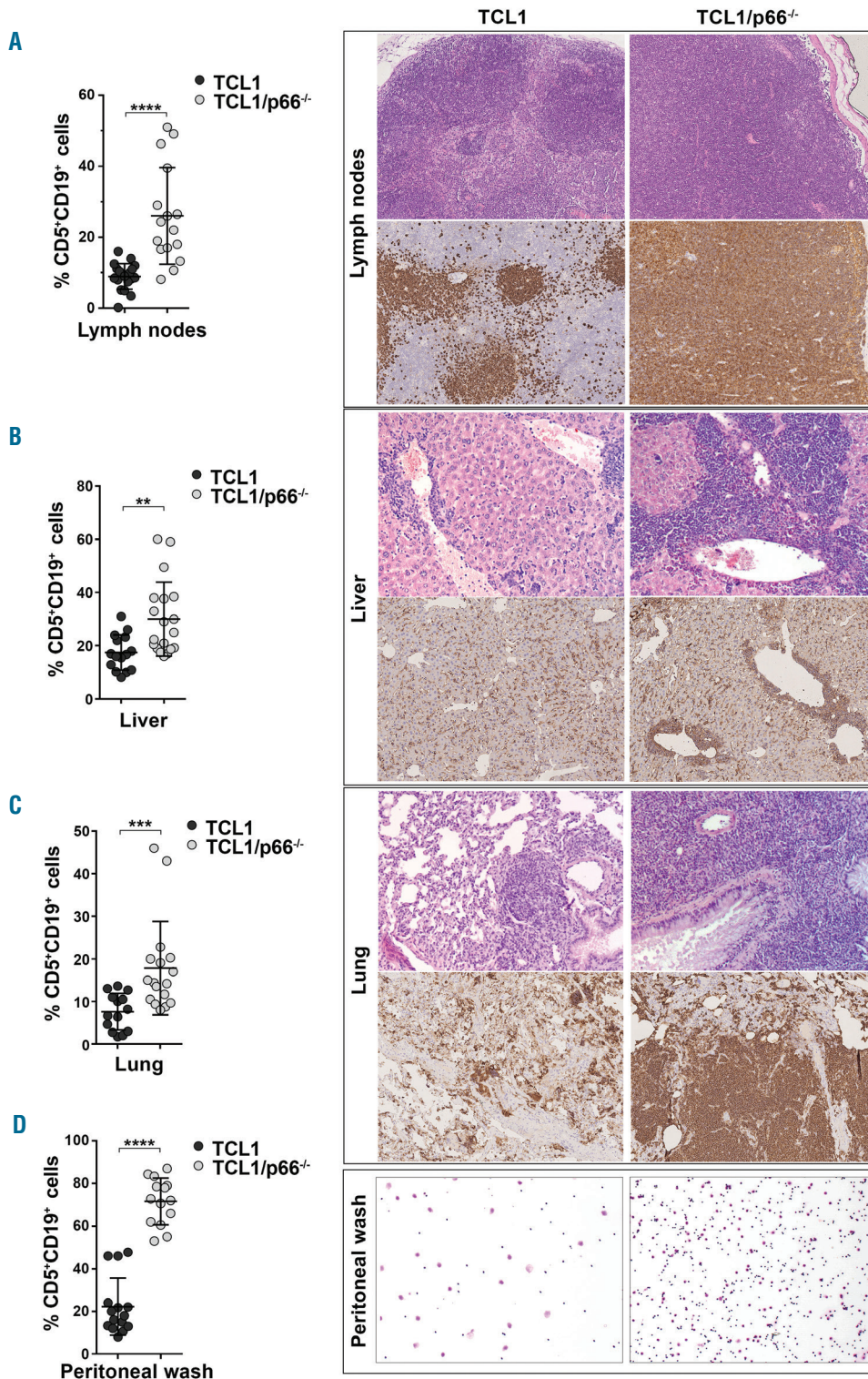
Lymphocyte homing to non-lymphoid organs is controlled by G protein-coupled receptors. B-cell homing to the liver is regulated by CCR1, CCR2, and CXCR3, while CCR2, CCR5, and CXCR3 have been implicated in B-cell homing to the lung.<sup>29-34</sup> CCR1 and CCR5 mRNA levels in leukemic cells were comparable 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<sup>-/-</sup> cells



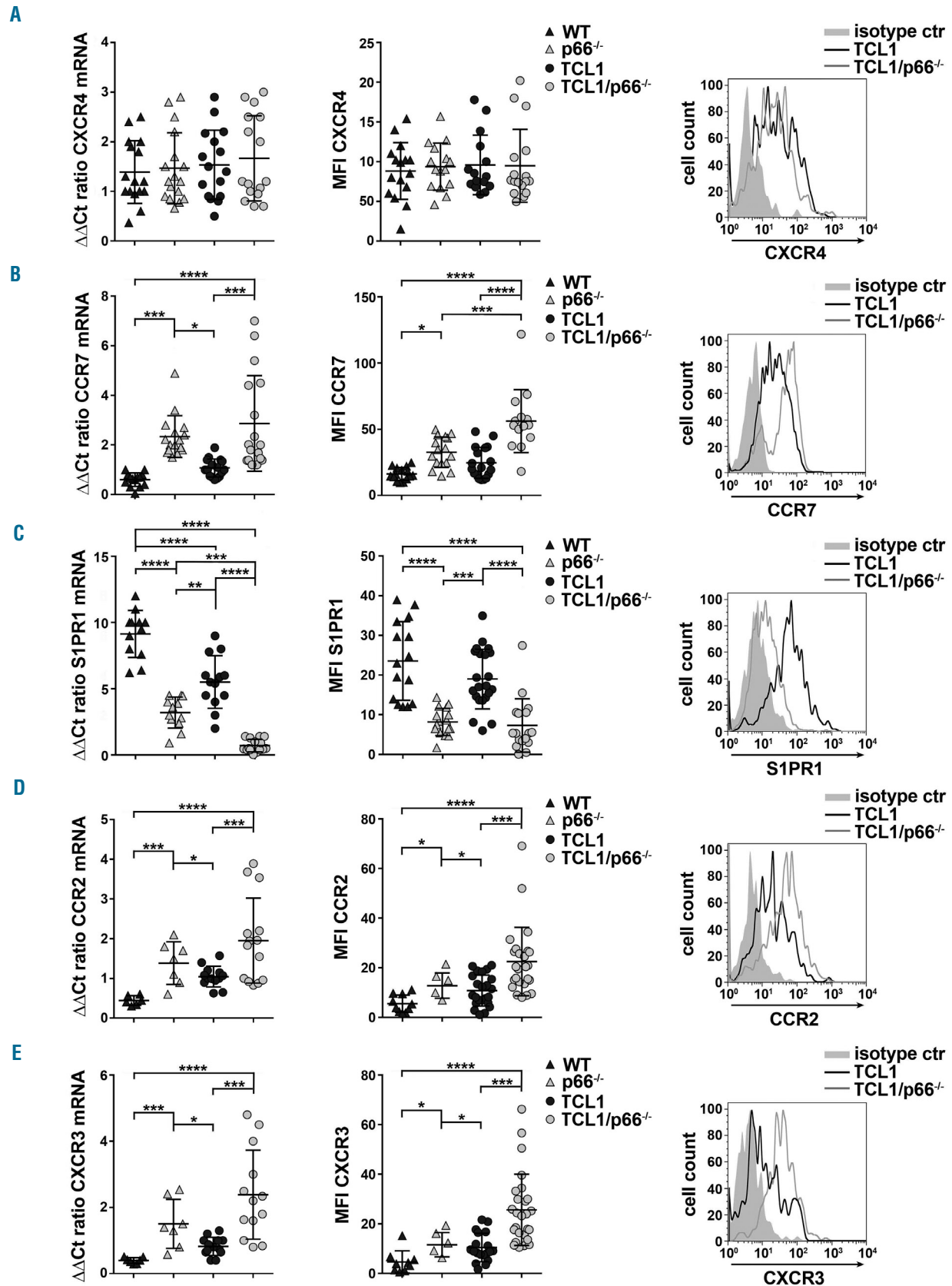
**Figure 3. p66Shc deficiency in leukemic cells results in enhanced chemoresistance.** (A) Quantitative real-time polymerase chain reaction analysis of Bcl-2 and Bax mRNA in leukemic cells purified from either wildtype (WT) (n=7) or p66Shc<sup>-/-</sup> (n=7) mice and from Eμ-TCL1 (n=10) or Eμ-TCL1/p66Shc<sup>-/-</sup> (n=12) mice with overt leukemia. The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta Ct$  method. (B) Immunoblot analysis with anti-Bcl-2 (left) and anti-Bax (right) antibodies of postnuclear supernatants of leukemic cells purified from either WT (n=3) or p66Shc<sup>-/-</sup> (n=3) mice and from Eμ-TCL1 (n=3) or Eμ-TCL1/p66Shc<sup>-/-</sup> (n=3) mice with overt leukemia. The stripped filters were reprobed with anti-actin antibodies. (C) Flow cytometric analysis of the percentages of annexin V<sup>+</sup>CD5<sup>+</sup>IgM<sup>+</sup> cells in peripheral blood from either WT (n=9) or p66Shc<sup>-/-</sup> (n=8) mice and from Eμ-TCL1 (n=20) or Eμ-TCL1/p66Shc<sup>-/-</sup> (n=22) mice. Samples were treated with either dimethylsulfoxide (DMSO) or 35 μM fludarabine (flu) for 16 h at 37 °C. (D) Flow cytometric analysis of the percentages of CD5<sup>+</sup>CD19<sup>+</sup> leukemic cells in peripheral blood, treated with either DMSO or 35 μM fludarabine for 16 h at 37 °C. Mean ± standard deviation. One-way analysis of variance (ANOVA), multiple comparisons. \*\*\*\*P≤0.0001; \*\*\*P≤0.001; \*\*P≤0.01; \*P≤0.05; ns, not significant.

than in E $\mu$ -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 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells compared to leukemic E $\mu$ -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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>+</sup>CD19<sup>+</sup> cells in lymph nodes (A), liver (B), lung (C) and peritoneal wash (D) from either E $\mu$ -TCL1 (n=15) or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> (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 E $\mu$ -TCL1 (n=5) or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> (n=10) with overt leukemia. (Immunoperoxidase staining; original magnification, 5x, 10x and 20x). Mean  $\pm$  standard deviation. Mann-Whitney rank sum test. \*\*\*\*P $\leq$ 0.0001; \*\*\*P $\leq$ 0.001; \*\*P $\leq$ 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<sup>+</sup>CD19<sup>+</sup> cells purified from either wildtype (WT) (n=16) or p66Shc<sup>-/-</sup> (n=15) mice (B1a cells) and from E $\mu$ -TCL1 (n $\ge$ 16) or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> (n $\ge$ 16) mice with overt leukemia. The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta$ Ct 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  $\pm$  standard deviation. One-way analysis of variance (ANOVA), multiple comparisons. \*\*\*\*P $\le$ 0.0001; \*\*\*P $\le$ 0.001; \*\*P $\le$ 0.01; \*P $\le$ 0.05.

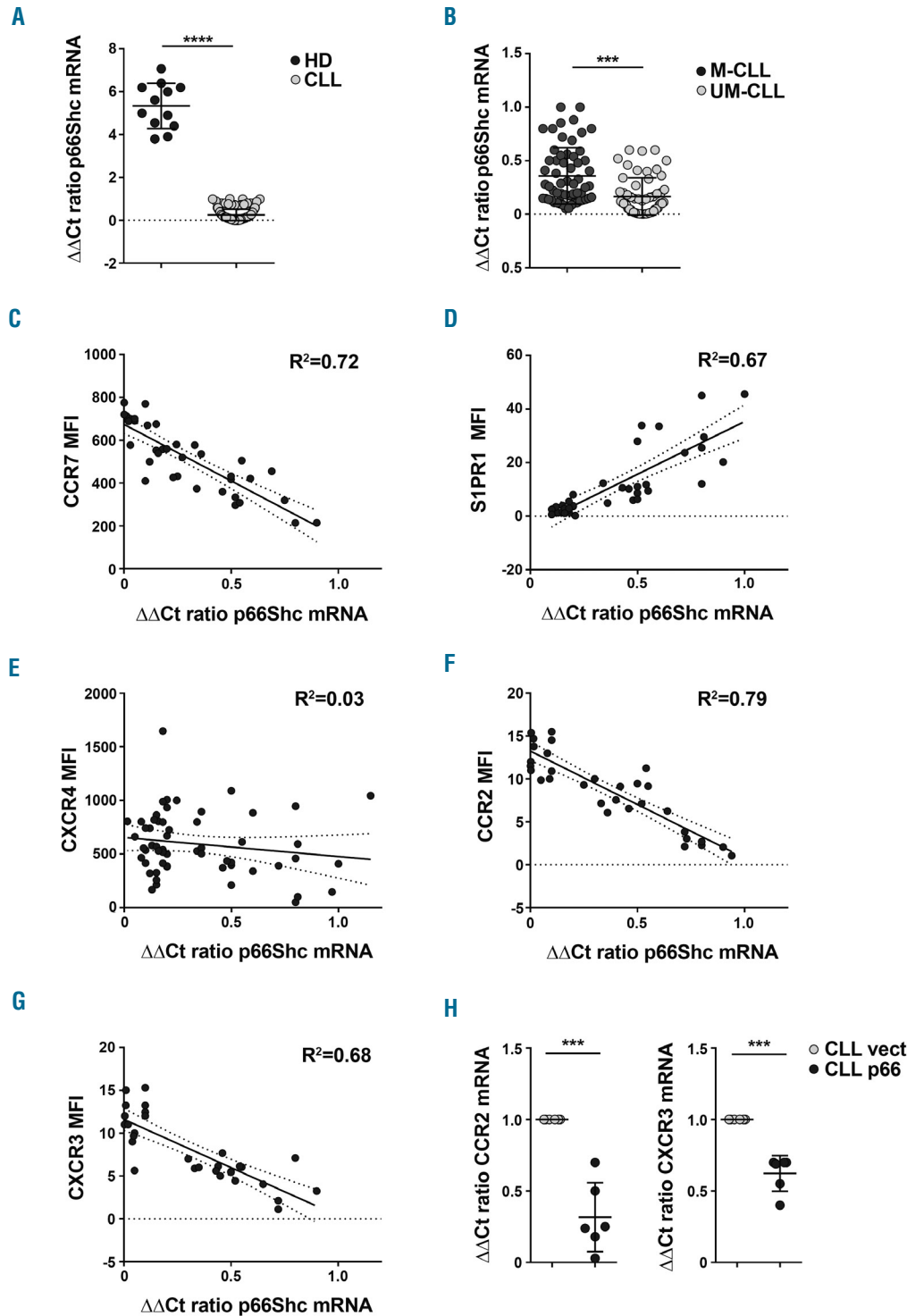


**Reconstitution of p66Shc in chronic lymphocytic leukemia cells normalizes their CCR2 and CXCR3 expression**

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,<sup>35</sup> compared to patients with mutated IGHV (M-CLL) (Figure 6A,B).<sup>6</sup> 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,<sup>9</sup> 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).



**Figure 6. p66Shc deficiency is associated with abnormal expression of chemokine receptors and lymphadenopathy in human chronic lymphocytic leukemia.** (A, B) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of p66Shc mRNA in B cells purified from either healthy donors (HD) (n=12) or patients with chronic lymphocytic leukemia (CLL) (n=157) (A), or B cells purified from CLL patients, grouped into those with mutated CLL (M-CLL) (n=67) or unmutated CLL (UM-CLL) (n=64) (B). (C-G) Correlation between mRNA levels of p66Shc and surface expression levels of CCR7 (C), S1PR1 (D), CXCR4 (E), CCR2 (F) and CXCR3 (G) in B cells purified from CLL patients (n≤89). (H) qRT-PCR analysis of CCR2 (left) and CXCR3 (right) mRNA in purified CLL B cells (n=6), nucleofected with either empty vector (CLL vect) or an expression construct encoding p66Shc (CLL p66). The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta\text{Ct}$  method. Mean  $\pm$  standard deviation. Mann-Whitney rank sum test. \*\*\*\* $P\leq 0.0001$ ; \*\*\* $P\leq 0.001$ .

Consistent with our finding that the upregulation of surface CXCR4 in CLL cells is mainly controlled post-translationally,<sup>11</sup> 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  $\Delta\Delta\text{Ct}$  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 who failed to

respond to ibrutinib therapy (Table 1 and *Online Supplementary Table S1*), 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.<sup>4</sup> We quantified homeostatic ROS production in CLL cells loaded with the cell-permeant probe CM-H<sub>2</sub>DCFDA. 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.<sup>6</sup> Furthermore, we found a direct correlation between ROS production and p66Shc expression in CLL cells (Figure 7B). These findings were recapitulated in CM-H<sub>2</sub>DCFDA-loaded E $\mu$ -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). ROS production was further impaired in E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells (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.<sup>8</sup> 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 $\rightarrow$ Q substitution at positions 132-133 (p66QQ), which disrupts cytochrome c binding (Figure 7D,E).<sup>8</sup> 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-H<sub>2</sub>DCFDA-loaded MEC1 transfectants showed enhanced ROS production in p66Shc-expressing cells, but not in cells expressing p66ShcQQ, compared to control cells (Figure 7F).

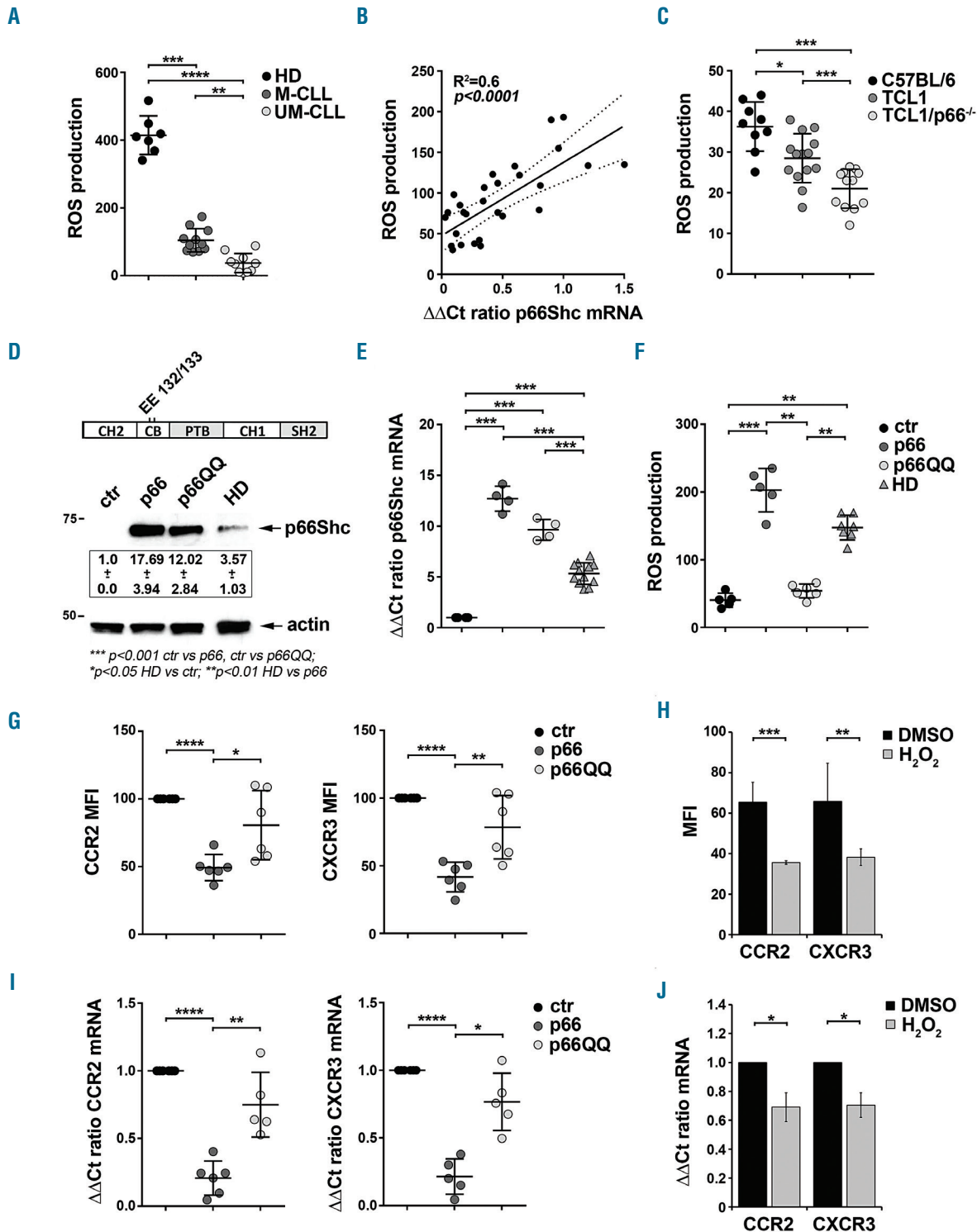
Mitochondrial redox signaling and apoptosis are also modulated by p53,<sup>36</sup> which is mutated in a large proportion of CLL patients<sup>1</sup> as well as in MEC1 cells.<sup>37</sup> To rule out a role for TP53 mutations in the enhanced ROS production by p66Shc-expressing MEC1 cells, ROS were measured in EBV-immortalized B cells, which express wildtype p53,<sup>38</sup> 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  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>, 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.**

	$\Delta\Delta\text{Ct}$ p66Shc mRNA	
	>0.24 $\pm$ 0.7 "above threshold"	$\leq$ 0.24 $\pm$ 0.7 "below threshold"
N. of CLL patients	30	34
N. of UM-CLL patients	6	19
% UM-CLL	20.00	55.88
N. of infiltrated lymph nodes	0.67 $\pm$ 0.23	2.27 $\pm$ 0.24 (*** $P$ <0.001 below vs. above threshold)
% LN >1.5 cm	53.33	88.23
% Spleen infiltration (> 13 cm)	13.33	70.59
% Liver infiltration (> 1 cm under arch)	3.33	29.41
	Patients responding to ibrutinib	Patients "failing" ibrutinib
Before CIT	0.8 $\pm$ 0.2	0.14 $\pm$ 0.06* (* $P$ <0.05 "failing" vs. responding patients)
Follow-up CIT	5.8 $\pm$ 2.3	0.35
Before ibrutinib	1.1 $\pm$ 1.04	0.12 $\pm$ 0.08
Follow-up ibrutinib	26.1 $\pm$ 6.9	0.23 $\pm$ 0.22** (** $P$ <0.01 "failing" vs. responding patients)

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  $\Delta\Delta\text{Ct}$  p66Shc mRNA; n CLL=157), or according to response to ibrutinib into either "responding" or "failing" based on International Working Group CLL response criteria.<sup>30</sup> Mann-Whitney rank sum test. \*\*\* $P$  $\leq$ 0.001; \*\* $P$  $\leq$ 0.01; \* $P$  $\leq$ 0.05. LN: lymph nodes; CLL: 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 E $\mu$ -TCL1 (n=13) or E $\mu$ -TCL1/p66Shc<sup>-/-</sup> (n=12) sick mice (C), loaded with CM-H<sub>2</sub>DCFDA. 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-H<sub>2</sub>DCFDA. 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  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Data refer to duplicate samples from four independent experiments. The relative gene transcript abundance was determined on triplicate samples using the  $\Delta\Delta Ct$  method. Mean  $\pm$  standard deviation. Mann-Whitney rank sum test. \*\*\*\* $P\leq 0.0001$ ; \*\*\* $P\leq 0.001$ ; \*\* $P\leq 0.01$ ; \* $P\leq 0.05$ . 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 E $\mu$ -TCL1 mice results in accelerated leukemogenesis and enhanced disease aggressiveness, with massive nodal and extranodal infiltration. The enhanced ability of leukemic p66Shc<sup>-/-</sup> 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 E $\mu$ -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<sup>-/-</sup> mice spontaneously develop age-related autoimmunity,<sup>5</sup> a feature frequently associated with CLL.<sup>1</sup> Interestingly, p66Shc downregulation in CLL B cells induces the expression of the inhibitory molecule ILT3,<sup>39</sup> suggesting that compensatory mechanisms might be operational to restrain CLL cell responses.

The negative impact of p66Shc deletion on disease progression and outcome in E $\mu$ -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.<sup>40</sup> The ROS-elevating activity of p66Shc<sup>41,44</sup> underlies this latter's ability to modulate the genes, several of which are redox-sensitive.<sup>41</sup>

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.<sup>3</sup> 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;<sup>8</sup> (ii) modulating CCR7 and CXCR4 by slowing down their endosomal recycling;<sup>7</sup> and (iii) attenuating CXCR4 and CXCR5 signaling by recruiting the phosphatases SHP-1 and SHIP-1 close to the activated receptors.<sup>9</sup> 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.<sup>7,8</sup> 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.<sup>8</sup> 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 E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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.<sup>21,22</sup> 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,<sup>42,43</sup> may account for the enhanced ability of leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> 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.*<sup>44</sup> for CXCR3). We show that p66Shc reconstitution in CLL cells reverts these abnormalities, validating in human CLL our finding that p66Shc deficiency contributes to CCR2 and CXCR3 overexpression in leukemic E $\mu$ -TCL1/p66Shc<sup>-/-</sup> cells.

p66Shc expression declines during disease progression in E $\mu$ -TCL1 mice, until its almost complete loss in mice with overt leukemia, paralleling the progressive decrease in fludarabine sensitivity of tumoral cells documented previously.<sup>20</sup> 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.<sup>45,46</sup> Although methylation increases in E $\mu$ -TCL1 mice during disease development,<sup>47</sup> it is unlikely that methylation of the p66shc promoter caused its progressive silencing, as p66Shc expression is not epigenetically silenced in B cells.<sup>6</sup> Rather, in these cells p66shc is transcriptionally regulated by STAT4, which is defective in CLL cells.<sup>10</sup> Interestingly, p66Shc can be restored both in CLL cells<sup>7</sup> and in leukemic E $\mu$ -TCL1 cells (Figure 1F,G) by treatment with ibrutinib, which also promotes STAT4 expression in leukemic E $\mu$ -TCL1 cells (Figure 1F,G). Ibrutinib modulates the expression of genes downstream of Btk in the BCR and CXCR4 pathways,<sup>48,49</sup> 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<sup>10</sup> 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 E $\mu$ -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.

## References

- Dighiero G, Hamblin TJ. Chronic lymphocytic leukaemia. *Lancet*. 2008;371(9617):1017–1029.
- Ortiz-Maldonado V, Mozas P, Delgado J. The biology behind B-cell lymphoma 2 as a target in chronic lymphocytic leukemia. *Ther Adv Hematol*. 2016;7(6):321–329.
- Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematol Am Soc Hematol Educ Progr*. 2011;2011(1):96–103.
- Giorgio M, Migliaccio E, Orsini F, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*. 2005;122(2):221–233.

5. Finetti F, Savino MT, Baldari CT. Positive and negative regulation of antigen receptor signaling by the Shc family of protein adaptors. *Immunol Rev.* 2009;232(1):115–134.
6. Capitani N, Lucherini OM, Sozzi E, et al. Impaired expression of p66Shc, a novel regulator of B-cell survival, in chronic lymphocytic leukemia. *Blood.* 2010;115(18):3726–3736.
7. Patrussi L, Capitani N, Cattaneo F, et al. p66Shc deficiency enhances CXCR4 and CCR7 recycling in CLL B cells by facilitating their dephosphorylation-dependent release from  $\beta$ -arrestin at early endosomes. *Oncogene.* 2018;37(11):1534–1550.
8. Capitani N, Patrussi L, Trentin L, et al. S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. *Blood.* 2012;120(22):4391–4399.
9. Patrussi L, Capitani N, Cannizzaro E, et al. Negative regulation of chemokine receptor signaling and B-cell chemotaxis by p66Shc. *Cell Death Dis.* 2014;5(2):e1068.
10. Cattaneo F, Patrussi L, Capitani N, et al. Expression of the p66Shc protein adaptor is regulated by the activator of transcription STAT4 in normal and chronic lymphocytic leukemia B cells. *Oncotarget.* 2016;7(35):57086–57098.
11. Patrussi L, Capitani N, Martini V, et al. Enhanced chemokine receptor recycling and impaired S1P1 expression promote leukemic cell infiltration of lymph nodes in chronic lymphocytic leukemia. *Cancer Res.* 2015;75(19):4153–4163.
12. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci U S A.* 2002;99(10):6955–6960.
13. Suljagic M, Longo PG, Bennardo S, et al. The Syk inhibitor fostamatinib disodium (R788) inhibits tumor growth in the E $\mu$ -TCL1 transgenic mouse model of CLL by blocking antigen-dependent B-cell receptor signaling. *Blood.* 2010;116(23):4894–4905.
14. Migliaccio E, Giorgio M, Mele S, et al. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature.* 1999;402(6759):309–313.
15. Stacchini A, Aragno M, Vallario A, et al. MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation. *Leuk Res.* 1999;23(2):127–36.
16. Patrussi L, Olivieri C, Lucherini OM, et al. p52Shc is required for CXCR4-dependent signaling and chemotaxis in T cells. *Blood.* 2007;110(6):1730–1738.
17. Yan X, Albesiano E, Zanasi N, et al. B cell receptors in TCL1 transgenic mice resemble those of aggressive, treatment-resistant human chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* 2006;103(31):11713–11718.
18. Dono M, Cerruti G, Zupo S. The CD5+ B-cell. *Int J Biochem Cell Biol.* 2004;36(11):2105–2111.
19. 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.
20. Johnson AJ, Lucas DM, Muthusamy N, et al. Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. *Blood.* 2006;108(4):1334–1338.
21. Kreiniz N, Beyar Katz O, Polliack A, Tadmor T. The clinical spectrum of hepatic manifestations in chronic lymphocytic leukemia. *clin lymphoma myeloma leuk.* 2017;17(12):863–869.
22. Carmier D, Dartigeas C, Mankikian J, et al. Serious bronchopulmonary involvement due to chronic lymphocytic leukaemia. *Eur Respir Rev.* 2013;22(129):416–419.
23. Pacini S, Pellegrini M, Migliaccio E, et al. p66SHC promotes apoptosis and antagonizes mitogenic signaling in T cells. *Mol Cell Biol.* 2004;24(4):1747–1757.
24. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and alpha4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. *Blood.* 2002;99(8):2977–2984.
25. Trentin L, Cabrelle A, Facco M, et al. Homeostatic chemokines drive migration of malignant B cells in patients with non-Hodgkin lymphomas. *Blood.* 2004;104(2):502–508.
26. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood.* 1999;94(11):3658–3667.
27. López-Giral S, Quintana NE, Cabrerizo M, et al. Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination. *J Leukoc Biol.* 2004;76(2):462–471.
28. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol.* 2005;23:127–159.
29. Akram IG, Georges R, Hielscher T, Adwan H, Berger MR. The chemokines CCR1 and CCR2 have a role in colorectal cancer liver metastasis. *Tumor Biol.* 2016;37(2):2461–2471.
30. Bigildeev AE, Shipounova IN, Svinareva DA, Drize NJ. Leukemia cells invading the liver express liver chemokine receptors and possess characteristics of leukemia stem cells in mice with MPD-like myeloid leukemia. *Exp Hematol.* 2011;39(2):187–194.
31. Zhao L, Lim SY, Gordon-Weeks AN, et al. Recruitment of a myeloid cell subset (CD11b/Gr1 mid) via CCL2/CCR2 promotes the development of colorectal cancer liver metastasis. *Hepatology.* 2013;57(2):829–839.
32. Rose CE, Sung S-SJ, Fu SM. Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. *Microcirculation.* 2003;10(3–4):273–288.
33. Jeyanathan M, Afkhami S, Khera A, et al. CXCR3 signaling is required for restricted homing of parental tuberculosis vaccine-induced T cells to both the lung parenchyma and airway. *J Immunol.* 2017;199(7):2555–2569.
34. Shetty S, Bruns T, Weston CJ, et al. Recruitment mechanisms of primary and malignant B cells to the human liver. *Hepatology.* 2012;56(4):1521–1531.
35. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood.* 1999;94(6):1848–1854.
36. Gào X, Schöttker B. Reduction-oxidation pathways involved in cancer development: a systematic review of literature reviews. *Oncotarget.* 2017;8(31):51888–51906.
37. Pozzo F, Dal Bo M, Peragine N, et al. Detection of TP53 dysfunction in chronic lymphocytic leukemia by an in vitro functional assay based on TP53 activation by the non-genotoxic drug Nutlin-3: a proposal for clinical application. *J Hematol Oncol.* 2013;6:83.
38. Allday MJ, Sinclair A, Parker G, Crawford DH, Farrell PJ. Epstein-Barr virus efficiently immortalizes human B cells without neutralizing the function of p53. *EMBO J.* 1995;14(7):1382–1391.
39. Zurli V, Wimmer G, Cattaneo F, et al. Ectopic ILT3 controls BCR-dependent activation of Akt in B-cell chronic lymphocytic leukemia. *Blood.* 2017;130(18):2006–2017.
40. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. *Leukemia.* 1996;10(3):456–459.
41. Hildeman DA, Mitchell T, Aronow B, Wojciechowski S, Kappler J, Marrack P. Control of Bcl-2 expression by reactive oxygen species. *Proc Natl Acad Sci U S A.* 2003;100(25):15035–15040.
42. Marra F, Tacke F. Roles for chemokines in liver disease. *Gastroenterology.* 2014;147(3):577–594.e1.
43. Belchamber KBR, Donnelly LE. Macrophage dysfunction in respiratory disease. *Results Probl Cell Differ.* 2017;62:299–313.
44. Trentin L, Agostini C, Facco M, et al. The chemokine receptor CXCR3 is expressed on malignant B cells and mediates chemotaxis. *J Clin Invest.* 1999;104(1):115–121.
45. Ventura A, Luzzi L, Pacini S, Baldari CT, Pelicci PG. The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. *J Biol Chem.* 2002;277(25):22370–22376.
46. Pezzicoli A, Olivieri C, Capitani N, Ventura A, Pelicci P, Baldari CT. Expression in T-cells of the proapoptotic protein p66SHC is controlled by promoter demethylation. *Biochem Biophys Res Commun.* 2006;349(1):322–328.
47. Chen S-S, Raval A, Johnson AJ, et al. Epigenetic changes during disease progression in a murine model of human chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* 2009;106(32):13433–13438.
48. Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood.* 2016;128(12):1609–1613.
49. Seda V, Mráz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur J Haematol.* 2015;94(3):193–205.
50. Eichhorst B, Fink A-M, Bahlo J, et al. First-line chemoimmunotherapy with bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab in patients with advanced chronic lymphocytic leukaemia (CLL10): an international, open-label, randomised, phase 3, non-inferiority trial. *Lancet Oncol.* 2016;17(7):928–942.