Chronic lymphocytic leukemia development is accelerated in mice with deficiency of the pro-apoptotic regulator NOXA

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. In recent years, it has become apparent that CLL is strongly dependent on its microenvironment for survival and proliferation. The pro-survival effect of the micro-environment is mainly mediated by upregulation of anti-apoptotic factors such as BCL-XL and MCL-1 upon receiving stimuli from surrounding T cells and macrophages.[1] In addition to changes in expression of anti-apoptotic proteins, we and others have reported altered expression of BH3-only pro-apoptotic NOXA and BMF proteins.[2,3] We have also found that the NOXA/MCL-1 balance in CLL cells is inverted in the lymph node compared to peripheral blood, which is indicative of an increase in chemoresistance.[4,5]

NOXA is a pro-apoptotic member of the BH3-only subfamily of Bcl-2 proteins, which also contains BID, BIM, BAD, and PUMA.[6] In contrast to BIM or PUMA, genetic ablation of NOXA does not result in an overt phenotype.[7] This is a reflection of the weak pro-apoptotic potential of NOXA itself. NOXA's main function is to bind to the anti-apoptotic protein MCL-1 and target it for proteasomal degradation.[8] This sensitizes the cell to the action of other BH3-only family members. NOXA may, therefore, be considered to be involved in fine-tuning the threshold of apoptosis.

Indeed, *Noxa*$^{-/-}$ mice do show a particular phenotype when exposed to antigens, revealing a role for NOXA in refining the response of B cells and T cells to antigens.[9,10] *Noxa*$^{-/-}$ mice show a low affinity B-cell response, due to a less stringent selection of clones that enter the germinal center.[10] Considering NOXA's role in shaping the B-cell response, and that CLL is expected to be driven by BCR-signaling, we hypothesized that NOXA expression levels determine the kinetics of CLL progression. In order to investigate this possibility, the consequences of NOXA loss in CLL were studied by crossing TCL1 transgenic mice, which develop CLL-like disease,[11] with *Noxa*$^{-/-}$ mice.

In view of past findings in CLL patients, in whom the NOXA/MCL-1 balance was found to be different between the peripheral blood and lymph nodes,[4] the peripheral blood and spleen of TCL1 mice with overt CLL-like disease were analyzed for expression of NOXA and MCL-1. In the absence of reliable antibodies to detect murine NOXA protein, NOXA mRNA levels in TCL1 CLL cells were determined by quantitative polymerase chain reaction, which showed a significantly lower expression of NOXA in CD5$^+$CD19$^+$ splenocytes than in CD5$^+$CD19$^+$ peripheral blood lymphocytes (Figure 1A). Conversely, levels of MCL-1 protein were significantly higher in CD5$^+$CD19$^+$ splenocytes than in CD5$^+$CD19$^+$ lymphocytes in peripheral blood, lymph node, and bone marrow (Figure 1B). These data indicate that the same dichotomy that is present in human CLL also exists in the TCL1 mouse model.

When crossed with TCL1 mice, ablation of NOXA does not seem to alter the phenotype of the emerging CLL cells in the peritoneal cavity, showing similar percentages and numbers of CD19$^+$B220^{dim}, CD5$^+$CD19$^+$CD11b$^+$CD43$^+$B220^{dim} (B1a) and CD5$^-$CD19$^+$CD11b$^+$CD43$^+$B220^{dim} (B1b) cells (Figure 1C,D). Examined both by immunofluorescence microscopy and intracellular flow cytometry, the amount of Ki67$^+$ cells, representative of dividing cells, was not dif-

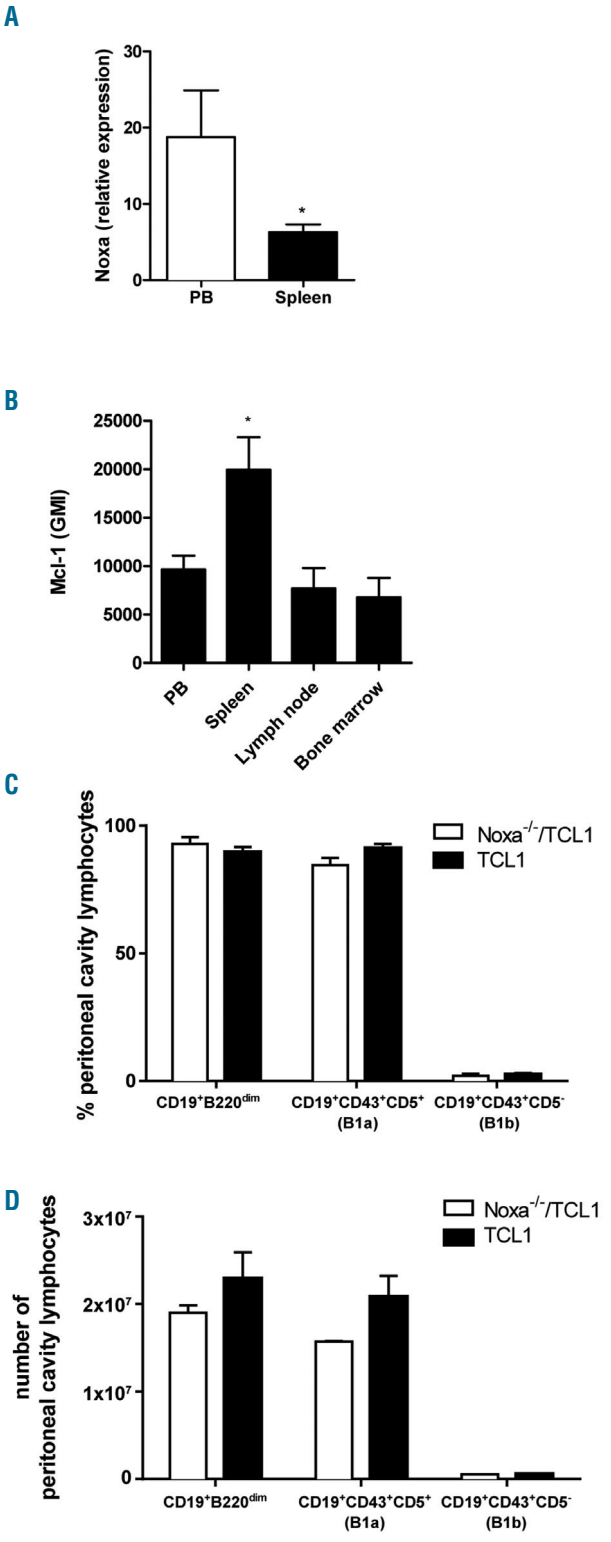

Figure 1. NOXA and MCL-1 are differentially expressed in TCL1 mice, ablation of NOXA does not change the phenotype of TCL1 CLL cells. (A) Comparison of mRNA levels in CD19$^+$CD5$^+$ cells in the peripheral blood and spleen of TCL1 mice (n=3). (B) Comparison of MCL-1 protein levels determined by flow cytometry in different organs of TCL1 mice (n=5). On average 460,000 CD5$^+$CD19$^+$ cells were analyzed in these mice. (C) Analysis of lymphocytes present in the peritoneal cavity of TCL1 (n=3, 9 months old), and *Noxa*$^{-/-}$/TCL1 (n=3, 8 months old) mice. Cells were stained for B1a markers (CD19$^+$CD43$^+$CD11b$^+$CD5$^+$B220^{dim}) and B1b markers (CD19$^+$CD43$^+$CD11b$^+$CD5$^-$B220^{dim}). The percentages of CD19$^+$B220^{dim} cells are also shown. (D) The same analysis as performed in (C) showing absolute numbers of peritoneal lymphocytes. *P<0.05.

ferent in the spleens of *Noxa*$^{-/-}$/TCL1 and TCL1 mice at 6 months of age (Figure 2A,B). However, the amount of apoptotic cells, as determined by immunostaining for cleaved caspase-3, present in the spleens of *Noxa*$^{-/-}$/TCL1 mice tended to be lower than that in TCL1 mice (Figure 2C,D). *Noxa*$^{-/-}$/TCL1 mice do not show any difference in the expression of apoptosis-related genes except for Noxa expression itself (*Online Supplementary Figure S1*).

A comparison of spleens from 7-month old TCL1 and *Noxa*$^{-/-}$/TCL1 animals indicated that *Noxa*$^{-/-}$/TCL1 mice had a higher percentage of CLL cells (Figure 3A,B). When mice succumbed to disease, the spleens of both TCL1 mice and *Noxa*$^{-/-}$/TCL1 mice showed features reminiscent of Richter transformation (*data not shown*). The absolute number of CD5^{+}CD19^{+} cells in the peripheral blood of *Noxa*$^{-/-}$/TCL1 mice (n=25) was significantly increased at 9 months of age (Figure 3C). The accelerated accumulation of CLL cells led to a decreased survival of *Noxa*$^{-/-}$/TCL1 mice compared to TCL1 mice (n=25) (Figure 3D) (342 days *versus* 396 days, respectively; $P<0.001$) as well as a decreased CLL-free survival (192 *versus* 240 days, respectively; $P<0.05$, *Online Supplementary Figure S2*). In agreement with a role for NOXA in counter-selection of low-affinity clones,[10] at 4 months of age B cells in the spleens of *Noxa*$^{-/-}$/TCL1 mice showed increased polyclonality whereas age-matched TCL1 B cells were already clonal. At 14 months of age this difference had disappeared and both types of mice showed one or two clones that dominated the B-cell population (Figure 3E).

NOXA is generally not considered to be a tumor suppressor in its own right, as loss of NOXA does not result in tumor development, but our data suggest that in the context of CLL it may function as an oncomodulator. This would be consistent with its described ability to induce apoptosis in oncogene-expressing cells[12] and with a role in potentiating irradiation-induced lymphoma.[13] The increased number of CLL cells in *Noxa*$^{-/-}$/TCL1 mice at 9 months of age compared to the number in TCL1 mice may be the result of impaired clonal deletion, which was also observed when *Noxa*$^{-/-}$ mice were immunized.[9] In TCL1 mice the leukemic population starts as a polyclonal population but culminates in the outgrowth of a much more restricted population of clones. Thus, in the setting of *Noxa*$^{-/-}$/TCL1 mice, a larger variety of clones survive and consequently there is a greater likelihood that an aggressive TCL1-driven clone emerges. These data suggest that a therapeutic strategy could be to target NOXA, either directly by interfering with its function or expression, or indirectly by targeting its binding partner MCL-1. Recently, MCL-1-specific BH3 mimetics have been described which may hold promise in this regard.[14] Already, the BTK-inhibitor ibrutinib provides an effective means to drive CLL cells from their protective environment in the lymph node,[15] which is also predicted to

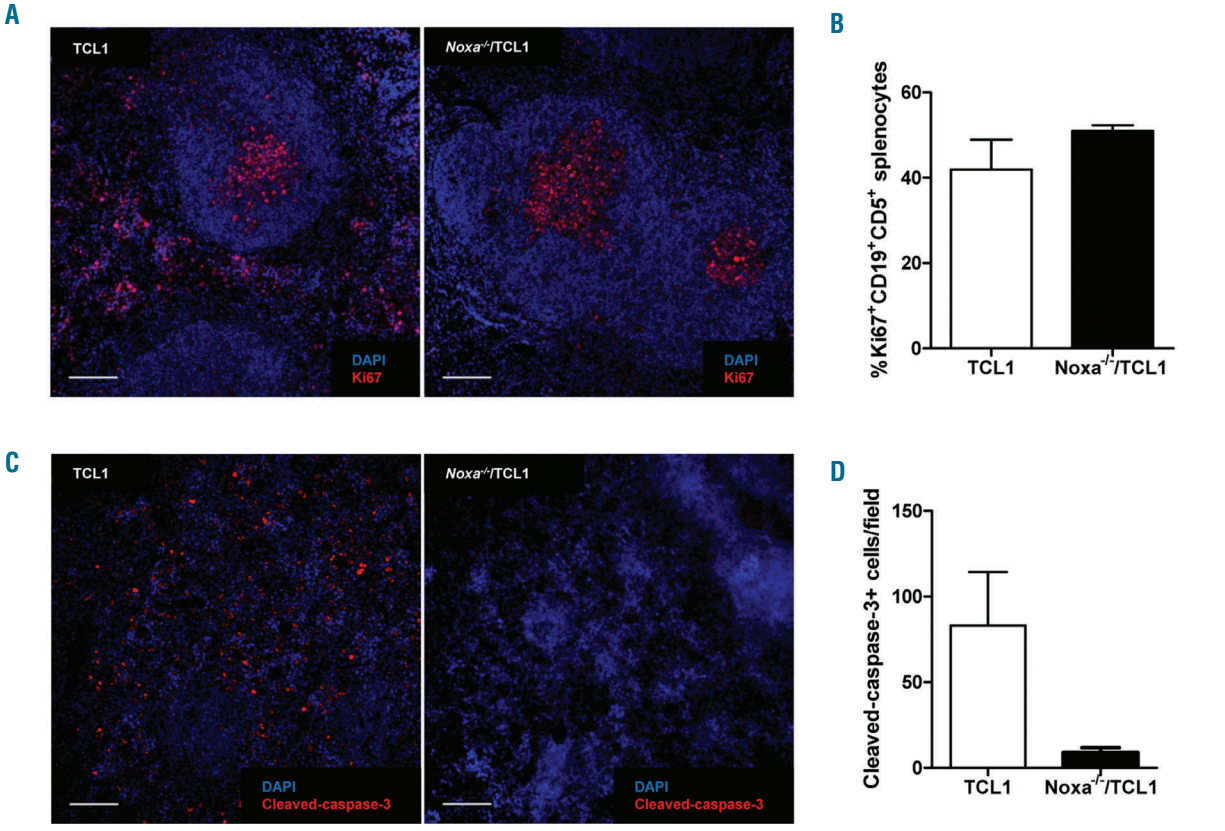

Figure 2. NOXA controls apoptosis in TCL1 mice. (A) Staining of splenic Ki67^{+} cells (depicted in red) in 6-month old TCL1 and *Noxa*$^{-/-}$/TCL1 mice. DAPI was used as a counterstain. Bar indicates 100 μm. (B) Analysis of CD19^{+}CD5^{+} splenocytes from the same TCL1 mice and *Noxa*$^{-/-}$/TCL1 mice by flow cytometry, (n=3, $P=0.27$). (C) Apoptotic splenocytes from 6-month old *Noxa*$^{-/-}$/TCL1 and TCL1 mice were stained for the presence of cleaved caspase-3 (red). Bar indicates 100 μm and DAPI was used as a counterstain (n=5, $P=0.17$). (D) Quantification of cleaved caspase-3-positive cells by double-blind counting of cells. Four different fields were counted from five different mice per group.

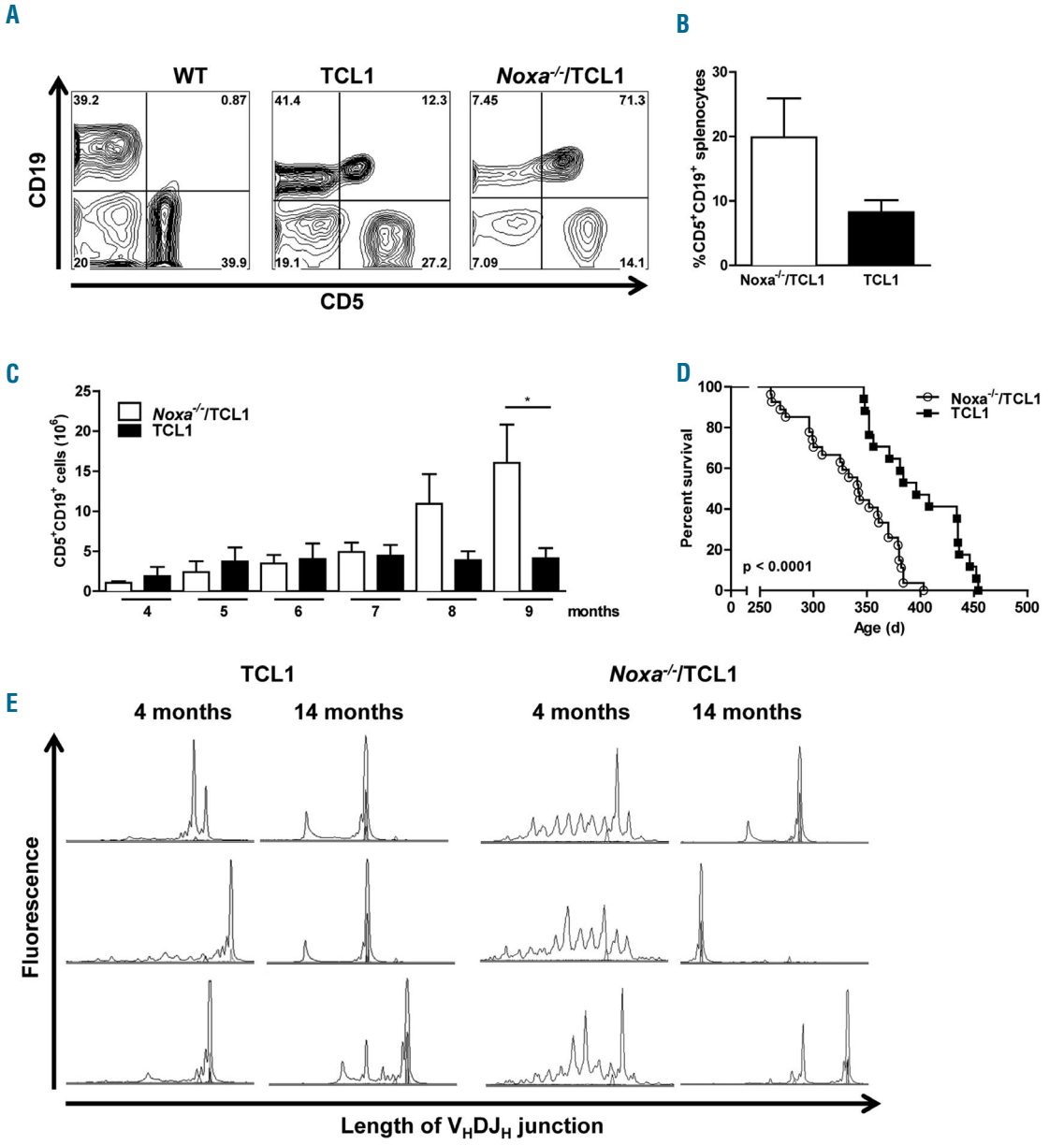

Figure 3. *Noxa*$^{-/-}$/TCL1 mice show accelerated CLL development and decreased survival. (A) Representative analysis of 7-month old wild-type (WT), TCL1 and *Noxa*$^{-/-}$/TCL1 spleens for the presence of CLL cells (CD5$^+$CD19$^+$ cells). (B) Comparison of spleens from 7-month old *Noxa*$^{-/-}$/TCL1 and TCL1 mice for CLL cells (n=3). (C) Monthly measurements of the amount of CD5$^+$CD19$^+$ cells present in peripheral blood of *Noxa*$^{-/-}$/TCL1 and TCL1 mice. The percentage of CD5$^+$CD19$^+$ cells was determined by flow cytometry and the absolute number of leukocytes by Coulter counter analysis of whole blood. From these data the absolute amount of CD5$^+$CD19$^+$ cells was calculated. (D) Survival analysis of Noxa$^{-/-}$/TCL1 and TCL1 mice, with median survival shown next to the respective curves. *P<0.05. (E) RNA from splenocytes of 4-month and 14-month old animals was used to assess B-cell receptor clonality. Spectra of individual mice at 4 months and 14 months are shown.

increase NOXA levels.[4] Furthermore, the sensitizing effect of NOXA to other pro-apoptotic signals may be exploited by combinations of the BCL2 inhibitor ABT-199 with methods to induce or stabilize NOXA protein levels (e.g. carfilzomib).

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