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Anti-leukemic activity of microRNA-26a in a chronic lymphocytic leukemia mouse model

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Running Title: Anti-leukemic activity of microRNA-26a in CLL

Abstract

The dysregulation of microRNAs (miRNAs) plays an important role in the pathogenesis of chronic lymphocytic leukemia (CLL). The E μ -*TCL1* transgenic mouse, which develops a leukemia similar to the aggressive form of human B-CLL, is a valuable model for testing novel therapeutic approaches. Here, we adopted this model to investigate the therapeutic effect of miR-26a, miR-130a, and antimiR-155.

To improve delivery of miRNA molecules into CLL cells, we developed novel anti-CD38-conjugated lipid nanoparticles, which were highly effective in delivering miRNA molecules into leukemic cells. Short and long-term experiments showed that miR-26a, miR-130a, and anti-miR-155 increased the levels of apoptosis after *in vitro* or *in vivo* treatment. In particular, *miR-26a* was the most effective in reducing leukemic cell expansion. At the end of long-term treatment, apoptosis was readily detectable by analysis of cleaved PARP and caspase-7. This effect could be directly related to miR-26a, as shown by the significant downregulation of its proven targets, the cyclin-dependent kinase 6 and Mcl1.

This study provides the first evidence that miR-26a could elicit an *in vivo* anti-leukemic activity through an increase in cell apoptosis. It also provides a novel *in vivo* approach that improves the efficiency and specificity of miRNA delivery into CD38+ leukemic cells.

Keywords: miRNA, CLL, Nanoparticles, CD38, miRNA delivery.

1 Introduction

2 Chronic lymphocytic leukemia (CLL) is the most common B-cell malignancy in Western countries. 3 CLL lymphocytes resemble memory B cells with a mature phenotype (1). This leukemia is characterized by the accumulation of CD5+ B lymphocytes in the blood, spleen, liver, lymph nodes, 4 and bone marrow. Despite its morphological homogeneity, CLL is a clinically heterogeneous 5 6 disease (2-4) with a number of adverse factors that affect the clinical course, including stage(5); CD38 positivity; unmutated variable region of the immunoglobulin heavy chain gene (IGHV) (6); 7 ZAP70 positivity (7); karyotype aberrations (8); and TP53, NOTCH1, SF3B1, and other gene 8 9 mutations (9, 10). Despite the progress in chemoimmunotherapy, CLL remains an incurable 10 disease (11).

To help decipher the pathogenic mechanisms of disease and evaluate the efficacy and 11 12 mechanisms of action of novel therapies, researchers have developed mouse models that resemble human CLL (12, 13). The Eµ-TCL1 transgenic (TCL1-tg) mouse develops a leukemia that is 13 highly similar to the aggressive form of human CLL (14-16). An important feature of TCL1 14 overexpression is that it exhibits 100% disease penetrance. At the age of 16-20 months, these 15 mice show a B-CLL-like disease characterized by spleno- and hepatomegaly associated with high 16 counts of white blood cells. Leukemias are characterized by clonal expansion of B cells with a 17 B220+/IgM+/CD5+ immunophenotype, unmutated IGHV, increased proliferation, and enhanced 18 19 AKT phosphorylation. Tumor cells in TCL1-tg mice have wild-type (WT) p53 and initially respond to fludarabine treatment (17). Notably, leukemic cells from a TCL1-tg donor can be transplanted by 20 intraperitoneal (IP) or intravenous injection into syngeneic WT or immunodeficient mice (e.g., 21 SCID) to accelerate the disease course and generate genetically homogeneous populations of 22 23 leukemic cells, which better allows for systematic comparison of novel therapies.

MicroRNAs (miRNAs) play a central role in the pathogenesis of CLL. They regulate gene expression 24 at the post-transcriptional level by targeting messenger RNA for degradation or translational 25 inhibition and thus can modulate several biological processes. As a consequence of their 26 27 deregulation, miRNAs may act as oncogenes or tumor suppressors (18). The discovery of the involvement of miRNAs in human cancer originated from studies of CLL (19). miR-15a and miR-16-28 1 were found within the minimal region of deletion at chromosome 13q, the most frequent 29 30 genetic alteration found in human CLL. Deletion of miR-15a/miR-16-1 in a knockout mouse model 31 was shown to confer predisposition to the development of an indolent form of leukemia similar to 32 human CLL (20). BCL2 was a target of these miRNAs; thus, their loss eliminated control over BCL2 expression (21). It is notable that venetoclax, a highly active and recently approved drug against 33 CLL, was designed to inhibit BCL2 activity (22). A prognostic signature consisting of a panel of 34 aberrantly expressed miRNAs has been identified in CLL (23). Other important miRNAs in human 35 CLL include miR-34a, which, being an effector of the p53 protein, is strongly downregulated in 36 17p-CLL cases, or miR-181b, which is downregulated during disease progression (24-26). In a more 37 38 recent comprehensive study, we found that a number of miRNAs are deregulated in CLL (27). miRNAs were differentially expressed between CLL cells and mature antigen-experienced B cells, 39 or correlated to specific pathological features (IGHV somatic mutations or specific cytogenetic 40 41 aberrations) or clinical parameters, such as time to first treatment (27). These studies established the groundwork from which miRNA-based therapies, either by restoring or repressing miRNA 42 activity, could be designed and tested. We have already investigated miR-181b as a potential 43 44 therapeutic molecule against leukemias of the TCL1-tg mouse model (28). The study proved that 45 this miRNA induced a measurable anti-leukemic effect. However, an improvement in delivery 46 methods was suggested.

Here, we used the Eμ-*TCL1*FL mouse model (15) to test the anti-leukemic activity of various miRNA
 mimics or anti-miRNA oligonucleotides (AMOs). To increase *in vivo* delivery, we developed a
 lipopolyplex formulation that included anti-CD38 conjugated lipid nanoparticles (CD38-NPs), which

4 considerably increased the specificity for hCD38+ leukemic cells of this mouse model. The choice

5 of CD38 as a molecule to target CLL cells was guided by its known importance in CLL (29), as well

- 6 as its structural characteristics (30) and the evidence of its antibody-mediated internalization (31).
- 7

8 Results

9 Leukemic cells of the Eµ-TCL1FL transgenic mouse are CD38+. Leukemic cells of the Eµ-TCL1FL transgenic mouse or transplanted into syngeneic FVB mice are characterized by B220+/CD5dim 10 surface markers (28). Here, we evaluated the expression of CD38. To this end, the splenic 11 12 lymphocyte population from transgenic or transplanted FVB mice with a high level of disease was 13 first sorted on the basis of physical parameters, and the B220+/CD5dim leukemic population was identified; finally, the lymphocytes within the B220+/CD5dim gate were analyzed for CD38 14 expression, showing that more than 98% of the cells were positive (Figure 1). This analysis not only 15 16 confirmed that the TCL1-tg model exhibits an immunophenotype typical of an aggressive form of B-CLL characterized by high expression of CD38, but it also provided a way to specifically target 17 leukemic cells. 18

19 Lipid nanoparticles conjugated with anti-CD38 antibodies can efficiently deliver miRNAs into CLL

cells. On the basis of this evidence, we developed lipid nanoparticles conjugated with anti-CD38
 antibodies (CD38-NPs) to improve the efficiency of *in vivo* miRNA delivery into leukemic cells. The

22 lipid NP-miRNAs were synthesized as described (32).

We investigated the efficiency of CD38-NPs both *in vitro* and *in vivo*. *In vitro*, we tested whether CD38-NPs could deliver mature miRNAs into murine *TCL1*-tg leukemic cells. We transfected cells with miR-181b mimics, miRNA that we had previously investigated (28). Twenty-four hours after transfection, cells were collected for RNA extraction and the levels of miR-181b measured (**Supplementary Figure 1**). These *in vitro* results indicated that CD38-NPs could efficiently deliver small RNA into leukemic cells and were approximately 2-fold more efficient than unconjugated NPs.

30 The TCL1-tg mouse represented an excellent model for testing in vivo delivery efficiency. We quantitatively investigated the delivery of 100 µg of miR-181b in different organs of FVB-TCL1 31 32 transplanted mice. Mice were treated when they exhibited a lymphocyte disease burden of 30-33 50% as the percentage of leukemic cells in peripheral blood. We tested CD38-NPs, non-conjugated 34 NPs, or Jet-PEI, a formulation used in our previous study (28), in 4 mice for each condition. The nanoparticles were administered by IP injection. After 24 hours, mice were sacrificed and organs 35 collected. Total RNA was isolated from liver, splenocytes, heart, kidneys, thyroid, lungs, muscle, 36 37 stomach, intestine, brain, and bladder and the expression levels of miR-181b were quantified by 38 digital PCR (Figure 2A). Spleen was the most efficiently targeted organ by CD38-NPs, followed by 39 liver. Compared with the physiological levels in these organs, the amount of miR-181b increased 40 more than 300-fold following CD38-NP-mediated delivery. In splenocytes, CD38-NPs induced an 41 18-fold increase compared with Jet-PEI and a 3-fold increase compared with non-conjugated NPs (Figure 2B). All other organs exhibited increased expression levels of miR-181b, generally at lower 42 43 levels and independent to the type of NP.

The greater delivery mediated by CD38-NPs in spleen and liver of diseased animals was likely attributable to the presence of a high number of CD38+ cells. This conclusion was supported by 1 the finding that no significant difference of miR-181b in spleen and liver was observed between

2 CD38-NPs and non-conjugated NPs delivery efficacy when experiment was performed in FVB WT

3 mice (**Supplementary Figure 2**). These findings indicated that CD38-NPs were capable of 4 improving efficiency of delivery and specificity of targeting for CD38+ CLL cells.

miR-26a, miR-130a, and anti-miR-155 are selected as potential anti-leukemic molecules. After
 the delivery efficacy of CD38-NPs was verified *in vivo* and *in vitro*, we tested the potential anti leukemic effects of a group of selected miRNAs/anti-miRNAs.

8 From the results of our previous microRNAome study in human CLL (27), we selected a group of 9 miRNAs that were deregulated in human CLL. Herein, we tested miRNA mimics miR-15, miR-16, 10 miR-26a, miR-125a, miR-130a, and miR-34a or AMOs of miR-21, miR-155, and miR-130a. A 11 scrambled RNA oligo was used as a negative control.

First, we investigated the *in vitro* biological activity on leukemic splenocytes isolated from the spleen of Eμ-*TCL1*FL transgenic mice. Leukemic splenocytes were transfected by the use of CD38-NPs and 48 hours after transfection, apoptotic activity was assessed by Annexin V assay (Figure **3A, supplementary figure 3)**. The highest apoptotic activity was detected in splenocytes treated with miR-26a, miR-125a, anti-miR-155 and miR-130a (p < 0.05). The efficiency of transfection of mimics/AMOs was assessed and confirmed in all the samples by analyzing the expression levels of each miRNA (Figure **3B**).

Next, for in vivo studies, we first evaluated if the CD38-NPs could also mediate a more effective 19 functional outcome than non-conjugated NPs. Given the high pro-apoptotic activity demonstrated 20 in vitro, we selected the miR-26a for measuring in vivo biological effects. 100 µg of miR-26a 21 22 mimics was delivered into mice with a high disease burden (30-50% TCL1+ cells in peripheral 23 blood) by using either CD38-NPs or non-conjugated NPs (3 mice in each group). As control, 3 mice 24 with a similar disease burden were treated with a scrambled RNA oligo. After 48 hours from 25 treatment (IP injection), mice were sacrificed and splenocytes isolated to analyze the biological 26 effects. As shown in Figure 4, in comparison with non-conjugated NPs, the use of CD38-NPs 27 induced a more effective down-regulation CDK6, a target of miR26a, and a clearer activation of 28 apoptosis, detectable by cleavage of PARP.

Based on these results, we evaluated a number of miRNA mimics/AMOs for their in vivo biological 29 30 activity using CD38-NPs as delivery vehicles. Transplanted mice with a high disease burden (30-50% TCL1+ cells in peripheral blood) were treated with 100 µg of different miRNA mimics or AMOs 31 32 (at least 3 mice in each group) by using CD38-NPs; as control, 4 mice with a similar disease burden 33 were treated with scrambled miRNA. After 48 hours of a single treatment (IP injection), mice were 34 sacrificed and splenocytes isolated to analyze the effects of miRNAs on apoptosis by the Annexin V 35 assay. As shown in Figure 5A, treatment with miR-26a or miR-130a mimics or with anti-miR-155 oligonucleotides induced a significant increase in apoptotic cells compared with control (p < 0.05), 36 37 while several other miRNAs did not exhibit significant differences. Notably, CD38-NP-Empty treated mice exhibited a negligible effect in comparison with control (p = 0.6). The efficiency of 38 39 delivery of mimic/AMOs in splenocytes was confirmed by quantitative PCR in all samples (Figure 40 5B). These results revealed the potential pro-apoptotic activity of miR-26a, miR-130a, and antimiR-155 against leukemic cells. 41

miR-26a reduces the expansion of leukemic cells of Eμ-TCL1 mice. Given the observed pro apoptotic effects after short-term treatments *in vitro* and *in vivo*, we sought to assess the
 potential anti-leukemic activity of miR-26a, miR-130a, or anti-miR-155 after 3 weeks of treatment.
 We performed the experiments by using FVB WT mice transplanted with Eμ-TCL1FL leukemic cells

as we have previously described (28). Syngeneic transplantations were performed in 6-week-old mice by IP injection of $5x10^5$ splenocytes, collected from an Eµ-*TCL1*FL transgenic mouse with advanced disease. We monitored the progression of leukemia in transplanted syngeneic mice over time by quantitatively measuring the increase of the human *TCL1* transgene, using a biomarker of transplanted leukemic cells, in DNA isolated from peripheral blood with digital PCR.

6 Mice were enrolled for treatment at about 8 weeks after transplantation, when leukemic cells 7 reached 15-30% of peripheral blood lymphocytes. At this stage, mice were assigned to 5 groups of 8 treatment with the same schedule of administration (IP injection of 100 μg of miRNA mimics or 9 AMOs 3 times a week for 3 weeks): (1) miR-26a, (2) miR-130a, (3) anti-miR-155, (4) scrambled 10 oligo control, and (5) CD38-NP-Empty nanoparticles. At the beginning of treatment, each group of 11 mice exhibited the same or a very similar arithmetic mean and standard deviation of leukemic 12 burden.

Before starting the treatments and 48 hours after the last treatment, we measured by digital PCR and FACS analysis the percentage of B220+/CD5+ leukemic cells present in the blood. Results of the two assays were in agreement. The differences between percentages of leukemic cells before and after treatment are shown in **Figure 6**. miR-26a treatment was the most effective in reducing the accumulation/expansion of leukemic cells in blood. The other molecules also induced a reduction of the leukemic cell burden in blood, albeit to a significantly less extent (**Figure 6**).

miR-26a promotes apoptosis in CLL cells. Given the significant anti-leukemic effect of miR-26a, we
 investigated the effect of miR-26a on leukemic splenocytes after 3 weeks of *in vivo* treatment.

The activation of apoptosis was clearly detectable by analysis of poly(ADP-ribose) polymerase (PARP) and caspase-7: the appearance of the 85-kD fragment (cleaved PARP), together with the strong increase in cleavage of caspase-7 were seen only in mice treated with CD38-NP-miR-26a, thus indicating the induction of apoptosis (Figure 7A).

To confirm that the apoptotic effect could be attributable to miR-26a activity, we analyzed the levels of two of its targets, Mcl1 and cyclin-dependent kinase 6 (Cdk6). Treatment with CD38-NPmiR-26a induced their downregulation, thus confirming the molecular activity of the delivered miR-26a (**Figure 7B**).

29

30 Discussion

The aim of this study was to demonstrate that miRNA-based therapies may have a place in the treatment of CLL, a human leukemia that is still incurable despite recent multiple successes. Our approach was based on evidence related to aberrant expressions of miRNA in CLL and on numerous observations (19, 23, 25, 27, 33).

To address the working hypothesis, we adopted the Eµ-*TCL1* mouse model, which develops a leukemia that is highly similar to an aggressive form of human CLL (15, 16). Using this model, we previously reported the anti-leukemic activity of miR-181b mimics (28). Here, we assayed miR-15a, miR-16-1, miR-26a, miR-125a, miR-130a, miR-34a, ant-miR-130a, anti-miR-21, and anti-miR-155. Among these, miR-26a, miR-130a, and anti-miR-155 induced apoptosis in leukemic cells both *in vitro* and *in vivo* in short-term assays. On the basis of these results, we tested the ability of miR-26a, miR-130a, and anti-miR-155 to counteract the *in vivo* accumulation/expansion of leukemic

- 42 cells of the E μ -*TCL1* mouse model.
- 43 miR-155 is a well-known oncomiR (34-39). In human CLL, a high level of miR-155 was associated 44 with various adverse prognostic factors (27, 40-43). Overexpression of miR-155 in transgenic mice

1 was shown to induce polyclonal B-cell expansion (34). The use of anti-miR-155 molecules has been 2 reported to significantly decrease in vivo tumor growth of BCWM1 cells derived from a patient with Waldenstrom macroglobulinemia (44). The role of miR-130a in tumorigenesis is, on the other 3 hand, controversial. Various reports have indicated either tumor-suppressive (45-51) or oncogenic 4 5 (52-56) activity, depending on the experimental settings. In human CLL, miR-130a was shown to 6 repress a survival autophagic pathway by targeting ATG2B and DICER1 (57). No animal models are available to examine the physiological or pathological function of miR-130a. Similarly, contrasting 7 results of dysregulated miR-26a have been reported in different tumor types (58-60). For example, 8 9 it was shown that miR-26a could facilitate glioblastoma formation in vivo (58, 61), but it was also shown that low miR-26a expression conferred a shorter overall survival in liver cancer patients 10 11 (62) and that its delivery through an adeno-associated viral vector could achieve a therapeutic effect on a MYC-induced liver cancer mouse model (59). In human CLL, a tumor suppressive 12 13 function of miR-26a was supported by evidence that its low expression was associated with a shortened time from diagnosis to first treatment (27), a clinical feature associated with poor 14 15 prognosis.

Despite the limited evidence linking miR-26a to CLL pathogenesis, this miRNA exhibited the 16 strongest activity against the accumulation/expansion of leukemic cells by enhancing apoptosis. 17 18 Several studies have previously documented that miR-26a is able to target and down-modulate 19 several protein-coding gene targets, including CDK6, cyclins D2/E2, and Mcl-1, in different tumor 20 cells (59, 63-65). Here, we found that a strong apoptotic effect was detectable in the spleen of treated mice and proof of miR-26a activity was confirmed by the downregulation of two of its 21 known targets, Mcl1 and Cdk6. The concomitant downregulation of the anti-apoptotic Mcl1 and 22 the cell cycle-promoting Cdk6 proteins suggests not only that miR-26a can promote apoptosis, but 23 24 that it can also inhibit CLL cell proliferation.

A second important result of this study was the development of nanoparticles conjugated with anti-CD38 antibodies that could efficiently deliver miRNA or AMOs into CD38+ cells, which typically characterize the most aggressive forms of CLL (29) and other hematological diseases such as myeloma. In fact, efficient, specific, and safe delivery of miRNA mimics or AMOs is a major challenge in miRNA-based therapeutic applications (66, 67).

30 Currently, methods for miRNA systemic delivery are mainly designed to target the liver (68, 69) 31 and they may use either viral (59, 70, 71) or non-viral systems (72-76). Significant therapeutic 32 effects in murine liver cancer models have been described (59, 70, 77). More difficult, however, is 33 the systemic delivery of miRNA mimics to other organs or tissues. Regarding hematopoietic cells, 34 in vivo delivery of antagomiR-126 was achieved in acute myeloid leukemia subpopulations by 35 using lipopolyplex nanoparticles conjugated with transferrin or antibody (anti-CD45.2) (72). The 36 chemical and physical characteristics of this formulation were shown to bypass hepatic uptake and ultimately achieve better delivery to hematopoietic organs (32). An analogous system based on 37 38 stable nucleic acid lipid vesicles conjugated with transferrin was developed to target multiple 39 myeloma cells expressing transferrin receptors (78, 79).

To develop a method for *in vivo* delivery to CLL cells, here we produced lipid nanoparticles, based on the formulation by Huang *et al* (32), conjugated with an anti-CD38 antibody, whose antigen is present on the surface of leukemic cells of Eμ-*TCL1* mouse model. Delivery to leukemic cells was superior to that of either non-conjugated nanoparticles or JetPEI, the nanosystem that we used in our previous report (28). The presence of the anti-CD38 antibody conferred specificity of action and also led to internalization of the nanoparticle complex in targeted spleen cells. The internalization was previously shown by using a panel of rat and mouse-anti CD38 mAbs (31). The

results indicated that the adopted methodology is efficient in transferring a cargo of different 1 2 miRNAs to leukemic cells. This competence was maintained in vivo. Spleen and liver were the most efficiently targeted organs by CD38-NPs. A small increase of miR-181b was also detectable in other 3 organs, especially when non-conjugated nanoparticles were used. Since diseased mice are 4 5 characterized by spleno- and hepatomegaly, these results strongly suggested that the CD38-NPs could increase delivery efficiency to these organs because they accumulate CD38+ CLL cells. The 6 7 characteristics of CD38 targeting in terms of safety were recently confirmed in vivo in mAbmediated therapy of human myeloma (80). 8

9 The main result of this study is that miR-26 exhibits a clear apoptotic effect on leukemic cells and a 10 significant reduction in leukemic cell expansion, suggesting its consideration for future CLL 11 therapy, possibly in combination with presently used therapeutic approaches. Moreover, we 12 provide a novel delivery method, which improves the efficiency and specificity of miRNA delivery 13 into CD38+ CLL cells. In humans, this approach could help target not only CLL cells in lymphatic 14 organs, but also other CD38+ B-cell malignancies, including various types of lymphoma, plasma 15 cell-derived neoplasms, and acute myeloid leukemia of the t(6;9) subtype (81-86).

16

17 Materials and Methods

Cell cultures and transfections. Mouse splenocytes were freshly isolated from diseased mice by using the procedure described by Bresin et al.(28). Transient transfections were performed with 100 nM pre-miR (Ambion) or single-stranded anti-miRNA (IDT) or scrambled negative controls complexed with lipidic nanoparticles (NPs or CD38-NPs). Cells were harvested 48 hours after transfection to evaluate apoptosis and miR expression.

23 Mice and syngeneic transplantation. The TCL1-tg mouse model used for these experiments has been previously described (15). Breeding pairs were provided to our group as a generous gift from 24 25 C. M. Croce (Ohio State University). FVB WT mice were obtained from Charles River Laboratories. 26 Mice had ad libitum access to water and a pellet diet. The animal room was maintained at 23°C on a 12-h light/12-h dark cycle. At the age of 6 weeks, the WT FVB female mice were transplanted by 27 IP injection of 5x10⁵ lymphocytes isolated from the spleen of an adult *TCL1*-tg mouse with 28 29 established leukemia. The engraftment of leukemic cells and the progression of disease were 30 monitored over time by the expression of B220/CD5 using flow cytometry analysis (FACS) or absolute quantification of the human TCL1 transgene by digital PCR of peripheral blood DNA. All 31 32 experiments with mice were conducted according to the 2010/63/EU directive of the European Parliament and Council. The protocol for animal experimentation was approved by the Italian 33 34 Ministry of Health (approval n. 40-2014 PR released on November 6, 2014).

Anti-CD38 monoclonal antibody. The anti-CD38 antibody is a rat monoclonal antibody (mAb) 35 specific for mouse CD38 antigen. It was produced in the laboratory of one of the authors (FM, 36 37 University of Turin). The NIMR-5 clone was expanded in vitro in Iscove's Modified Dulbecco's Medium and purified by high pressure liquid chromatography. The purified IgG was then sterilized 38 39 by 0.22-µm filtration (Millipore Polyethersulfone Millex-GP Syringe Filter Unit, radiosterilized) and 40 detoxified by Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific) (87). Specificity was 41 confirmed by reacting the purified mAb with the murine X63.Ag8 myeloma and analyzing binding by indirect immunofluorescence (87). 42

43 Preparation of nanoparticles. The lipid components of the nanoparticles were 1,2-dioleoyl-sn 44 glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol
 45 (MW ~2000; DMG-PEG; Avanti Polar Lipids, Alabaster, AL), and linoleic acid (Sigma-Aldrich, St.

Louis, MO). The molar ratio of DOPE:linoleic acid:DMG-PEG was 50:48:2. The preparation of empty nanoparticles was performed as previously described (32). The anti-CD38 antibody was conjugated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG2000 maleimide) according to the method described in a previous study (72) and was then post-inserted into the surface of lipopolyplex nanoparticles to form the CD38-NP-miRs.

6 In vivo treatment with mimics or AMOs. The synthetic mimics and anti-miRNAs for in vivo delivery 7 were purchased from Axolab (Germany). For short-term experiments, the FVB mice (at least 3 8 mice in each group) were enrolled for treatment when the disease reached 30-50% of TCL1positive cells in peripheral blood, and they were treated once with 100 µg of specific single-strand 9 10 mimics/anti-miRNA and sacrificed after 48 hours. For long-term experiments, the mice (at least 6 mice in each group) were enrolled for treatment when the disease reached 15-30% of TCL1-11 positive cells in peripheral blood, and they were treated with 100 µg of the specified molecules 3 12 times a week for 3 weeks. For long-term treatments, the levels of disease were measured by FACS 13 and droplet digital PCR (ddPCR) analysis the day before the start of treatment and 48 hours after it 14 15 ended.

16 Flow cytometry. Blood samples (20 μ L) were placed in a tube containing 0.5 M EDTA as an 17 anticoagulant. Erythrocytes were lysed by treatment with ammonium chloride (0.8%) and EDTA (0.1 mM) (Sigma). Cells were incubated with specific antibodies for 10 min in ice, washed, and 18 analyzed by flow cytometry in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, 19 California, USA) by using FlowJo software (TreeStar, Ashland, OR). Data for 5x10⁴ cells within the 20 lymphocyte light-scatter gate were collected. In these assays, color compensation was performed 21 22 before cell acquisition by using the MACS Comp Bead Kit, anti-rat Igk (Miltenyi Biotec, Gladbach, 23 Germany). During analysis, gates were set by using a Fluorescence Minus One control strategy. Leukemic cells were identified as B220+/CD5dim cells, normal B lymphocytes as B220+/CD5- cells, 24 25 and T lymphocytes as B220-/CD5+ cells. The following antibodies were used: FITC rat anti-mouse 26 CD5 (Cat. 553020, BD Pharmingen) and PeCy5 rat anti-mouse CD45R/B220 (Cat. 553091, BD 27 Pharmingen). CD38 expression on the surface of leukemic splenocytes was detected by triple staining using a PE rat anti-mouse CD38 antibody (Cat. 130-103-008, Miltenyi Biotec) together 28 with the FITC rat anti-mouse CD5 and the PeCy5 rat anti-mouse CD45R/B220 antibody indicated 29 30 above.

- Analysis of apoptosis. The apoptotic effect of mimic or anti-miR molecules on murine splenocytes
 treated *in vitro* or *in vivo* was assessed by the Muse[™] Annexin V and Dead Cell Assay kit (Cat.
 MCH100105, Merck) according to manufacturer's protocol. To count viable cells, we used the
 Muse Count & Viability Assay kit (Cat. MHC100102, Merck).
- RNA and DNA extraction. Total RNA and DNA were isolated from cells or tissues by using the
 Maxwell Rapid Sample Concentrator (RSC) Instrument (Promega) with the Maxwell RSC miRNA
 Tissue Kit and the Maxwell RSC Blood DNA Kit.
- Reverse transcription and ddPCR. The ddPCR method was used to measure the expression level 38 of miRNAs. The reverse transcription reaction was performed on 5 ng of total RNA by using the 39 40 TaqMan miRNA Reverse Transcription assay. After appropriate dilution, 1 µL of the cDNA was used for amplification in a 20-µL reaction volume containing ddPCR Supermix for Probes (Bio-Rad) and 41 42 the TaqMan miRNA PCR probe set. Droplets generation, cycling conditions for TaqMan assays and the counting of positive droplets were performed according to procedures described by Miotto et 43 44 al. (88). To normalize the relative abundance of miRNAs, we used the Taqman Assays for RNAs U6 45 or SNO412 (Applied Biosystems).

1 Monitoring leukemic cells by ddPCR. A ddPCR approach was used to measure the human TCL1 2 transgene in DNA extracted from peripheral blood cells of FVB transplanted mice. We used 3 primers/probes specific for the human TCL1 transgene (forward 5'-CTCTGGCTCTTGCTTCTTAG-3'; reverse 5'-CACCCGTAACTGTAACCTATC-3'; probe--/56-FAM/TCGTGTATT/Zen/TGGACGAGAAGCA 4 5 GCA/3IABkFQ/) and primers/probes specific for the mouse Gapdh gene as an endogenous reference (forward 5'-GGTGTGAACCACGAGAAATA-3'; reverse 5'-CTCATGGCAGGGTAAGATAAG-3'; 6 probe--/5HEX/ACAAC TTTG/Zen/GCATTGTGGAAGGGC/3IABkFQ/). The probes for human TCL1 and 7 mouse Gapdh were conjugated with FAM and HEX, respectively. The ddPCR assay was performed 8 9 on 10 ng of genomic DNA, using the same procedures as described above.

10 Western blot analysis. Splenocytes of treated and control mice were suspended in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich), supplemented with protease and 11 phosphatase inhibitors (Sigma-Aldrich), according to the manufacturer's protocol. Protein 12 concentrations were measured with the Bradford assay (Bio-Rad). Equal amounts (30 μ g) of 13 protein extracts from all samples were applied to SDS-PAGE electrophoresis and then transferred 14 to a PVDF membrane (Bio-Rad). The membrane was incubated with primary antibodies as follows: 15 anti-CDK6 (rabbit, Cat. sc177, Santa Cruz), anti-Mcl-1 (Rabbit, Cat.5453 Cell Signalling), anti-PARP 16 17 (rabbit, Cat. 9542, Cell Signalling), anti-caspase-7 (rabbit, Cat. 9492, Cell Signaling) 1:1000. β-18 Tubulin H235 (rabbit, Cat. sc9104, Santa Cruz), , was used as a normalized control. The membrane was incubated with anti-rabbit IgG, HRP-linked antibody (Cat. 7074, Cell Signaling). For signal 19 detection, ClarityTM Western ECL Substrate (Cat. 170-5060, Bio-Rad) was used according to the 20 manufacturer's instructions. Digital images were acquired with Chemidoc (BioRad). Signals were 21 quantified by ImageJ software and protein expression levels normalized according to β -tubulin 22 23 expression.

- 24
- 25 **Conflict of Interest:** The authors declare no competing financial interests.
- 26

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34 (http://www.nature.com/onc)

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1 2

FIGURES LEGENDS

3 Figure 1. In the Eµ-TCL1FL TG mouse, the leukemic population (B220+/CD5dim) is CD38+. Cells 4 were isolated from the spleen of an adult $E\mu$ -TCL1FL transgenic mouse (upper panels) or a transplanted FVB-TCL1 mouse (lower panels). The left panels show the lymphocyte population, the 5 6 middle panels the B220+/Cd5dim leukemic population. The right panels show the CD38+ 7 lymphocytes within the B220+/CD5dim population: more than 98% of the cells are positive.

8

9 Figure 2. Bio-distribution of miR-181 after the use of CD38-NPs as an *in vivo* delivery agent. (A) Bio-distribution 24 hours after intraperitoneal injection of miR-181b mimics (100 µg) delivered 10 into a transplanted FVB-TCL1 mouse with a high level of disease by the use of CD38-NP, regular 11 nanoparticles (NPs), or Jet-PEI. (B) Highlights of splenocytes and liver. Treatment with CD38-NPs 12 induces the most significant increase in the miR-181b level in splenocytes compared with other 13 14 tissues or methods of delivery (** p < 0.001, * p < 0.05, respectively), likely reflecting the high 15 number of CD38+ leukemic lymphocytes present in this organ. The results are normalized to 16 SNO412 expression.

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Figure 3. Pro-apoptotic activity of different miRNA mimics/AMOs in leukemic splenocytes in 18 vitro. (A) The apoptotic activity of miRNAs/anti-miRNAs in leukemic splenocytes was evaluated by 19 the Muse Cell Analyzer using the Muse Annexin V & Dead Cell Assay 48 hours after transfection. 20 Statistical assessment of each miRNA effect is referred to the Scramble control. *= p < 0.05 (t-21 22 test). (B) The level of each miRNA after transfection was assessed by ddPCR at the time when apoptosis measured. miRNA levels were normalized on SNO412 and non-transfected cells (UNT). 23

24

25 Figure 4. CD38-NPs mediate an in vivo more effective functional outcome than non-conjugated 26 NPs. Forty-eight hours after a single injection of miR-26a mimics (100 ug) with CD38-NPs or non-27 conjugated NPs, mice with established leukemia were sacrificed, splenocytes were isolated and 28 analyzed for RNA and protein expression. CD38-NP-miRNA26a produced a higher level of miR-26a than NP-miR26a. The results are shown as relative expression of miR-26a normalized to SNO412 29 30 expression, as measured by ddPCR. As a result, the treatment with CD38-NPs could mediate more 31 effective functional outcomes as revealed by a clearer apoptotic effect (cleavage of PARP) and a stronger down regulation of a miR-26a target (CDK6). 32

33

34 Figure 5. Apoptotic activity of miRNA/anti-miRNA molecules after a single in vivo treatment of 35 mice with established leukemia. Mice with established leukemia were sacrificed 48 hours after a 36 single treatment with miRNA/anti-miRNA molecules. Splenocytes were isolated to evaluate apoptosis. (A) Using an Annexin V assay, we observed a significant increase in the percentage of 37 apoptotic cells 48 hours after treatment with CD38-NP-miR-26a (p = 0.013), CD38-NP-miR-130a (p 38 39 = 0.03), or CD38-NP-anti-miR-155 (p = 0.002). (B) At the same time, the relative expression of miR-16, miR-26a, miR-155, miR-130a, miR-34a, and miR-21 was measured in splenocytes by using 40 41 ddPCR. The results are shown as the relative expression level, normalized to SNO412 expression.

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Figure 6. miR-26a exhibits the strongest activity against Eµ-TCL1FL leukemic cells. Percentages of 43 circulating leukemic cells were measured just before starting treatments and 48 hours after their 44 45 completion. The differences between these points were acquired and plotted for each mouse 46 enrolled in the study. miR-26a exhibited the strongest effect on expansion of leukemic burden, as 47 shown in the graph and by the p-values in the associated table. P-values < 0.05 are highlighted by 48 a grey background. N = number of mice, SD = standard deviation.

1

Figure 7. Modulation of pro-apoptotic proteins and direct targets of miR-26a *in vivo.* Forty-eight hours after the end of long-term treatments, mice were sacrificed and the spleen collected. Proteins were extracted from splenocytes of CD38-NP-miR-26a-treated and control mice for Western blot analysis. (A) Cleaved PARP and cleaved caspase-7 are evidence of apoptosis and were clearly visible in the samples treated with miR-26a, but not in the controls. (B) The direct molecular activity of miR-26a was demonstrated by showing that two of its known targets, Mcl1 and Cdk6, were down-modulated in the samples treated with miR-26a, but not in the control.

- 10
- 11



Figure 1













Difference Posts										
		Leukemia Burden Difference post-pre			t-tests					
	Ν	Mean	SD		mR-26a	miR-130a	anti-miR-155	Ctrl	CD38-NP-empty	
mR-26a	10	23.1	3.2	\parallel	1.00E+00	8.30E-03	1.00E-01	2.60E-03	2.00E-04	
miR-130a	7	34.5	1.7		8.30E-03	1.00E+00	5.32E-01	2.49E-01	1.36E-02	
anti-miR-155	6	32.0	3.7		1.00E-01	5.32E-01	1.00E+00	1.83E-01	2.61E-02	
Ctrl	9	38.6	3.0		2.60E-03	2.49E-01	1.83E-01	1.00E+00	2.53E-01	
CD38-NP-empty	6	43.6	2.5		2.00E-04	1.36E-02	2.61E-02	2.53E-01	1.00E+00	

