Antineoplastic effects of liposomal short interfering RNA treatment targeting *BLIMP1/PRDM1* in primary effusion lymphoma

RNA interference (RNAi) has been suggested to represent a promising therapeutic approach in different disease settings. Primary effusion lymphoma (PEL) is a plasmablastic lymphoma consistently expressing B lymphocyte-induced maturation protein 1 (Blimp-1), a pivotal transcriptional regulator during terminal differentiation of B cells into plasma cells. Here we report, for the first time, that transient knockdown of the *BLIMP1* gene (also known as PR Domain Containing 1 with ZNF Domain, or *PRDM1*) using small interfering RNA (siRNA) delivered by liposomes, induced remarkable killing in PEL cell lines. Furthermore, in a murine model of PEL, significantly prolonged survival was achieved by intraperitoneal treatment with such anti-*BLIMP1* lipoplexes, while no vector-induced toxicity was observed. This effective and safe RNAi strategy, based on liposomal siRNA targeting a master transcription factor of post-germinal center B cells, may indeed be a potential treatment against plasmablastic lymphoma.

Over the last decade, after the discovery of RNAi as a useful experimental tool for selective gene silencing, the possibility of harnessing siRNA also in disease treatment has been increasingly investigated.[1-4] With regard to cancer treatment, several preclinical studies have shown promising therapeutic potential associated with siRNA-mediated down-regulation of different tumor-relevant genes, both in solid neoplasms and hematologic malignancies. However, clinical applications of RNAi seem to be dependent on the use of optimal delivery systems. To date, while there are still safety concerns regarding siRNA-expressing viral vectors, the use of liposomal carriers has been suggested to be a safe and effective option, protecting siRNA from rapid degradation after administration, as well as efficiently delivering them into target cells.[3,4] Recently, two clinical trials have provided major proof of concept for cationic liposome-mediated RNAi therapy, targeting abnormal genes implicated in transthyretin amyloidosis and hypercholesterolemia.[5,6]

PEL is an aggressive B-cell lymphoma, characterized by a plasma cell-like gene expression profile,[7] driven by human herpesvirus-8/Kaposi sarcoma associated herpesvirus (HHV8/KSHV). PEL typically arises in serous body cavities of immunocompromised patients (mainly those infected with human immunodeficiency virus) or elderly subjects; it is manifested by pleural or peritoneal malignant effusions and has a poor prognosis.[8] Indeed, it is often not feasible to administer standard chemotherapy or systemic antivirals (e.g. Cidofovir) to such fragile patients, who frequently have comorbidities and impaired organ function, and novel therapeutic strategies are, therefore, required for PEL. Interestingly, Godfrey et al. first proposed an RNAi-based approach to treat PEL, showing effective PEL inhibition *in vitro* and *in vivo* by lentiviral vectors encoding short hairpin RNA able to knockdown HHV8/KSHV-associated oncogenes.[9] Aiming to expand RNAi strategies for PEL treatment, we previously tested different lipid-based nanocarriers for their ability to target PEL cell lines efficiently.[10-12] In the present work, we investigated the *in vitro* and *in vivo* antineoplastic activity associated with liposomal siRNA-mediated knockdown of the *BLIMP1/PRDM1* gene, which encodes Blimp-1, a transcription factor considered a crucial regulator of the transcriptional network in post-germinal center B-cell stages.

For *in vitro* experiments, PEL cell lines (BCBL-1, HBL-6 and CRO-AP/3) were cultured in fetal calf serum-supplemented RPMI 1640 medium, as described elsewhere,[13,14] and were treated with siRNA against *BLIMP1/PRDM1* (siRNA sequences in the *Online Supplementary Information*) complexed with liposomes to form anti-*BLIMP1* siRNA lipoplexes (formulated and characterized as previously described).[10-12] The same liposomes, either loaded with scrambled oligonucleotides (i.e. mock siRNA, commercially provided together with validated siRNA) or empty (vehicle), as well as free anti-*BLIMP1* siRNA without a vehicle, were used as negative controls. PEL viability and cell concentration were assessed daily by staining with annexin V/propidium iodide (Miltenyi Biotech, Bergisch Gladbach, Germany), in accordance with the manufacturer's instructions, and by using an AcT8 automated cell counter (Beckman Coulter Inc., Brea, CA, USA), respectively. Caspase-3 activity was assayed (Calbiochem, EMD Biosciences, San Diego, CA, USA) as previously reported,[15] to detect the activation of the apoptotic pathway. The cell cycle was analyzed using a commercial BrdU/7-AAD assay (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction and western blot assays were performed, as previously described,[15] to evaluate mRNA levels and protein expression, respectively, for Blimp-1 and other relevant B-cell transcription factors.

To investigate putative antineoplastic effects associated with *BLIMP1* silencing in PEL and, at the same time, to define the most suitable carriers for such RNAi therapy, we preliminarily performed a screening set of *in vitro* experiments (*data not shown*), using different lipid-based formulations to deliver anti-*BLIMP1* siRNA (50-200 nM) into PEL cell lines, and then we tested PEL viability at 24, 48 and 72 h after treatment, by using the annexin V/propidium iodide assay. Compared with controls, most formulations of anti-*BLIMP1* siRNA-lipid lipoplexes were able to cause increased cell death in all PEL cell lines tested. In particular, we found that a single treatment with anti-*BLIMP1* siRNA (100 nM), delivered by dioleoyl trimethylammonium propane (DOTAP) liposomes (i.e. anti-*BLIMP1* siRNA/DOTAP lipoplexes, 1:100 molar ratio; mean encapsulation efficiency 85%, mean diameter 402±31 nm, polydispersivity index 0.13±0.02, ζ-potential 19±1 mV; transfection efficiency 60-80%), induced the most remarkable and consistent reduction of PEL viability and cellularity, while only mild toxicity was associated with the use of empty DOTAP liposomes (10 μM) (Figure 1, and *Online Supplementary Figures S1* and *S2*). Of note, at 48 and 72 h after such treatment, we observed a mean decrease in PEL viability of 83% and 95%, as well as a mean cell loss equal to 79% and 91% of the number of cells treated, respectively (Figure 1A,B). All these results were statistically significant when compared with related controls (ANOVA test, $P<0.001$). In line with these findings, activation of caspase-3 (*Online Supplementary Figure S3*) and accumulation in the sub-G1 phase (*data not shown*) were detected in PEL cells treated with anti-*BLIMP1* siRNA/DOTAP lipoplexes, without evidence of cell-cycle arrest in G1 or G2 phase (no increase of either G1/S or G2/S ratios between treated cells and controls), thus suggesting that Blimp-1 inhibition induced apoptosis directly rather than having a cytostatic effect. In parallel, the effective knockdown of *BLIMP1* after liposomal siRNA treatment was confirmed by quantitative real-time polymerase chain reaction analysis and western blot assays, showing a specific decrease in *BLIMP1* transcrip-

tion levels and protein expression, respectively (Figure 1C,D), while no consistent modifications of other relevant B-cell transcription factors (namely *BCL6, PAX5* and *CIITA3*; *data not shown*) were observed. Taken together, these findings demonstrate that transient silencing of *BLIMP1* by liposomal siRNA is sufficient to rapidly induce PEL killing, revealing that this transcription factor is strictly required for PEL survival. Interestingly, our data are in agreement with the results of an *in vitro* study on multiple myeloma, reporting that the infection of myeloma cell lines with lentiviral vectors expressing anti-*BLIMP1* short hairpin RNA may directly cause tumor apoptosis, without triggering a process of de-differentiation or reprogramming the molecular network of neoplastic plasma cells.[15]

We next tested the therapeutic potential of anti-*BLIMP1* siRNA/DOTAP lipoplexes *in vivo*, using a previously described PEL xenograft murine model.[14] Briefly, 24 severe combined immunodeficiency (SCID)/CB17 mice were intraperitoneally (i.p.) injected with 25x10^6 CRO-

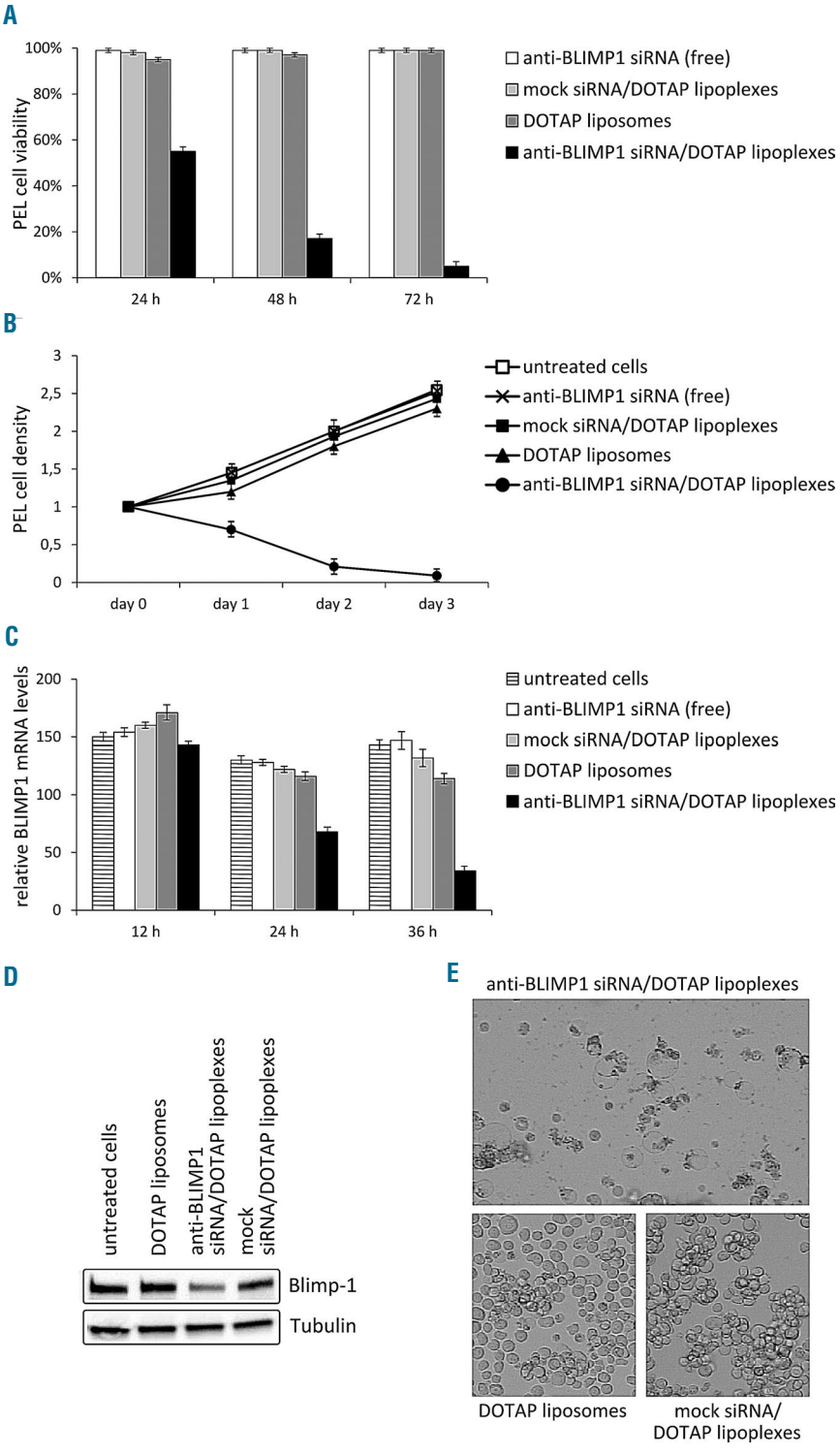

Figure 1. *In vitro* antineoplastic effect of *BLIMP1* silencing by anti-*BLIMP1* siRNA/DOTAP lipoplexes. (A) Proportion of viable PEL cells (BCBL-1) after treatment with DOTAP liposomes delivering anti-*BLIMP1* siRNA (i.e. anti-*BLIMP1* siRNA/DOTAP lipoplexes), empty DOTAP liposomes, mock siRNA/DOTAP lipoplexes, or free anti-*BLIMP1* siRNA (without vehicle) as assessed by annexin V-propidium iodide assay at three different time-points. Results are expressed as relative percentages, calculated on viable cell fractions detected in untreated cells at the same time-points (% viability in treated cells / % viability in untreated cells, x 100). Data represent mean values of three independent experiments, performed in triplicate wells for each condition. Error bars represent standard error (SE) of the mean. Similar data were obtained with HBL-6 and CRO-AP/3 cells (*Online Supplementary Figures S1* and *S2*, respectively). (B) Modifications in PEL cellularity over time, after treatments as in panel A. Results are expressed as fold variations of the density of PEL cells at day 0. Data represent mean values obtained in three independent experiments, performed in triplicate wells for each condition. Error bars represent SE of the mean. (C) Relative expression of *BLIMP1* transcripts was determined by real-time reverse transcriptase polymerase chain reaction in BCBL-1 cells at 12, 24 and 36 h, after the treatments indicated above. Data are reported as mean ± SD. (D) Western blot panel shows Blimp-1 protein expression at 48 h in untreated BCBL-1 cells, in cells exposed to empty DOTAP liposomes, or treated with either anti-*BLIMP1* or mock siRNA/DOTAP lipoplexes. After treatment with anti-*BLIMP1* siRNA/DOTAP lipoplexes, the relative quantification of Blimp-1 protein was reduced to 0.41, using untreated cells as the reference (Image Lab 5.1, Bio-Rad Laboratories, Hercules, CA, USA). (E) Light microscopy (magnification 200x) of BCBL-1 cells shows marked cell death at 48 h after treatment with anti-*BLIMP1* siRNA/DOTAP lipoplexes (upper panel), compared to cells treated with empty DOTAP liposomes or mock siRNA/DOTAP lipoplexes (lower panels).

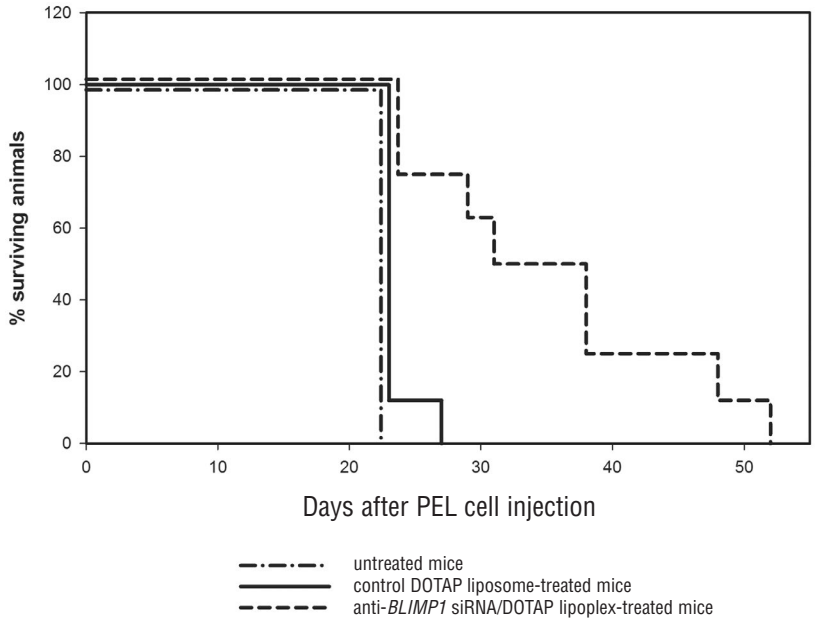

Figure 2. ***In vivo*** **antineoplastic effect of anti-*BLIMP1* siRNA/DOTAP treatment. Kaplan-Meier survival curves for CRO-AP/3-injected SCID mice treated with anti-*BLIMP1* siRNA/DOTAP lipoplexes or empty DOTAP liposomes, and for untreated animals. Data were obtained from two separate experiments of 12 animals each (4 mice per group). *BLIMP1* siRNA/DOTAP-treated mice showed a statistically significant increase in survival compared to control mice (log-rank test, *P*=0.002).**

AP/3 cells, and i.p. treated or mock–treated on days 1, 2 and 3 after tumor induction. Animals were divided into three groups: one group received DOTAP liposomes loaded with anti-*BLIMP1* siRNA (1.2 nmol/mouse/day), one control group received empty DOTAP liposomes and another control group was untreated. Mice were monitored daily for the development of ascites, by measuring body weight gain and abdominal distension, and survival studies were performed.[14] This study was evaluated and approved by the local ethical committee (CEASA, n. 66698/2012); as suggested, we minimized the number of control groups.

Survival curves obtained in two independent experiments are reported in Figure 2. All control, untreated mice (8 of 8) and mock-treated animals (empty DOTAP liposomes, 8 of 8) developed lymphomatous ascites and were culled by day 23 and 27, respectively (median survival in both groups, 23 days). The treatment with anti-*BLIMP1* siRNA/DOTAP lipoplexes significantly increased the overall survival time (median survival, 34 days) of treated animals (log-rank test, anti-*BLIMP1* siRNA/DOTAP lipoplexes *versus* empty DOTAP liposomes, *P*=0.002), even if lethal lymphomatous ascites eventually developed in all cases. These data indicate that anti-*BLIMP1* siRNA were able to exert marked anti-tumor activity also *in vivo*, while DOTAP liposomes alone were well tolerated and did not delay PEL growth. Here, by using a "pre-emptive" treatment approach, which indeed was likely to reduce the incipient, high ($25x10^6$cells) tumor dose, we obtained prolonged survival associated with long-lasting prevention of PEL outgrowth. As previously discussed,[9] the treatment of full-blown ascites in murine models of PEL remains a challenge, and will require more intensive therapy with anti-*BLIMP1* siRNA/DOTAP possibly in combination with standard chemotherapy.

In conclusion, these preclinical observations provide evidence that a safe liposomal siRNA treatment targeting Blimp-1 may be effective against PEL, and, conceptually, that silencing a master transcription factor of post-germinal center B cells exerts remarkable antineoplastic activity against a plasmablastic lymphoma. Thus, this work may offer the rationale for testing such an approach in PEL patients, as well as for exploring similar RNAi-based therapy in other lymphoproliferative diseases. Additional studies are warranted to address other interesting issues of *BLIMP1* inhibition, such as putative effects against normal plasma cells as well as on the HHV8/KSHV life cycle.

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References

1. Bumcrot D, Manoharan M, Koteliansky V, Sah DWY. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol. 2006;2(12):711–719.
2. Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010;464(7291):1067-1070.
3. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. 2009;8(2):129-138.
4. Ozpolat B, Sood AK, Lopez-Berestein G. Liposomal siRNA nanocarriers for cancer therapy. Adv Drug Deliv Rev. 2014;66:110-116.
5. Coelho T, Adams D, Silva A, et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N Engl J Med. 2013;369(9):819-829.
6. Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, et al. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet. 2014;383(9911):60-68.
7. Jenner RG, Maillard K, Cattini N, et al. Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. Proc Natl Acad Sci USA. 2003;100(18):10399-10404.
8. Carbone A, Gloghini A. KSHV/HHV8-associated lymphomas. Br J Haematol. 2008;140(1):13-24.
9. Godfrey A, Anderson J, Papanastasiou A, Takeuchi Y, Boshoff C. Inhibiting primary effusion lymphoma by lentiviral vectors encoding short hairpin RNA. Blood. 2005;105(6):2510-2518.
10. Ruozi B, Riva G, Belletti D, et al. Immunoliposomal systems targeting primary effusion lymphoma: in vitro study. Nanomedicine (Lond). 2010;5(7):1051-1064.
11. Ruozi B, Riva G, Belletti D, et al. Cidofovir-loaded liposomes: an intro-study using BCBL-1 cell line as a model for primary effusion lymphoma. Eur J Pharm Sci. 2010;41(2):254-264.
12. Belletti D, Riva G, Tosi G, et al. Novel polymeric/lipidic hybrid systems (PLHs) for effective cidofovir delivery: preparation, characterization and comparative in vitro study with polymeric particles and liposomes. Int J Pharm. 2011;413(1-2):220-228.
13. Luppi M, Trovato R, Barozzi P, et al. Treatment of herpesvirus associated primary effusion lymphoma with intracavity cidofovir. Leukemia. 2005;19(3):473-476.
14. Calabrò ML, Gasperini P, Di Gangi IM, et al. Antineoplastic activity of lentiviral vectors expressing interferon-alpha in a preclinical model of primary effusion lymphoma. Blood. 2009;113(19):4525-4533.
15. Lin FR, Kuo HK, Ying HY, Yang FH, Lin KI. Induction of apoptosis in plasma cells by B lymphocyte-induced maturation protein-1 knockdown. Cancer Res. 2007;67(24):11914-11923.