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Robust and cost-effective CRISPR/Cas9 gene editing of primary tumor B cells in Eμ-TCL1 model of chronic lymphocytic leukemia

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Ability to genetically edit primary B cells via CRISPR/Cas9 technology represents a powerful tool to study molecular mechanisms of B-cell pathogenesis. In this context, employing ribonucleoprotein complexes (RNPs), formed by recombinant Cas9 and genome-targetting single guide RNA molecules, brings in advantage of accelerated set-up and protocol robustness. Gene editing via RNP electroporation has been recently applied to primary tumor cells isolated from patients chronic lymphocytic leukemia (CLL), suggesting an efficient and valuable tool for studying leukemic cell biology and biomarker validation.[1,2] The work by Nardi et al. on this topic proposed to electroporate unmanipulated primary CLL cells that are subsequently put in culture with human CD40L-expressing fibroblasts and soluble stimuli to promote CLL cell proliferation. In this context, cellular proliferation is required to achieve homozygous gene editing, whereas in unstimulated CLL cells it is possible to achieve only the heterozygous editing.[1] The method published by Mateos-Jaimez et al. relies on the preactivation of CLL cells with CD40L/BAFF/IL-21-expressing stromal cells, followed by RNP electroporation and continuation of the stimulatory coculture.[2] Both methods approach 80%–90% of editing efficiency and allow to perform downstream in vitro experiments on edited leukemic cells.

Application of a similar RNP-based editing approach to the widely used murine model of CLL, the Eμ-TCL1 transgenic mice,[3] represents a valuable and versatile tool to explore CLL biology in vivo. Examples illustrating its feasibility has been first shown in studies by Chakraborthy et al. and Martines et al.[4,5] The published method consists in preactivating primary CD19+CD5+ leukemic B cells by TLR9 agonist CpG ODN-1668, followed by RNP electroporation and intraperitoneal injection of 30×10^6 electroporated cells to promote expansion of edited leukemic cells in vivo. Despite this method has been proven effective, it has not been set up to expand edited TCL1 cells in vitro. This is associated with high experimental costs and does not allow to perform functional analysis of gene editing phenotype

prior to the in vivo transfer, which eventually becomes not feasible if edited cells are unfit in vivo.

Hence, we envisioned a new approach that would allow to expand RNP-electroporated TCL1 cells in vitro prior to transfer. To this end, we first optimized culture conditions for TCL1 cells evaluating their viability and proliferation after treatment with different stimuli. We observed that ODN-1668 stimulation, although being efficient in activating TCL1 cells in the short-term,[5] does not allow to expand them in vitro (Supporting Information S1: Figure S1). We thus evaluated different stimulation conditions and established that coculture of TCL1 cells with irradiated fibroblasts expressing murine CD40L was sufficient to induce proliferation of leukemic cells over 5 days of culture (Figure 1A,B). Curiously, IL-4 and IL-21 addition did not enhance CD40-driven TCL1 cell growth, in contrast to what is typically observed for human CLL cells (Figure 1A).

Next, we optimized electroporation settings for TCL1 cells using an in vitro translated mRNA encoding GFP[1] and an electroporation system with bimodal pulsing strategy previously employed by us for human CLL cell electroporation.[1] Among 13 tested electroporation conditions, the optimal one allowed us to maintain >90% vitality of recovered TCL1 cells and achieve around 80% cell transfection efficiency (Figure 1C; electroporation settings in Supporting Information S1: Table S1; gating strategy in Supporting Information S1: Figure S2). To set up a protocol for CRISPR/Cas9 gene editing of TCL1 cells, we followed a pipeline indicated in Figure 1D. Electroporating TCL1 cells with polyglutamic acid-stabilized RNPs[7] and allowing them to proliferate 4–5 days post-electroporation typically allowed us to achieve >80% gene KO in bulk population, either with single (Figure 1E,F) or double combination of sgRNAs (Figure 1G,H and Supporting Information S1: Figure S3). For example, targeting the surface marker CD19 proved convenient as a control that could be easily assessed by flow cytometry (Figure 1G,H). The protocol typically allowed for three-fold expansion of electroporated TCL1 cells,

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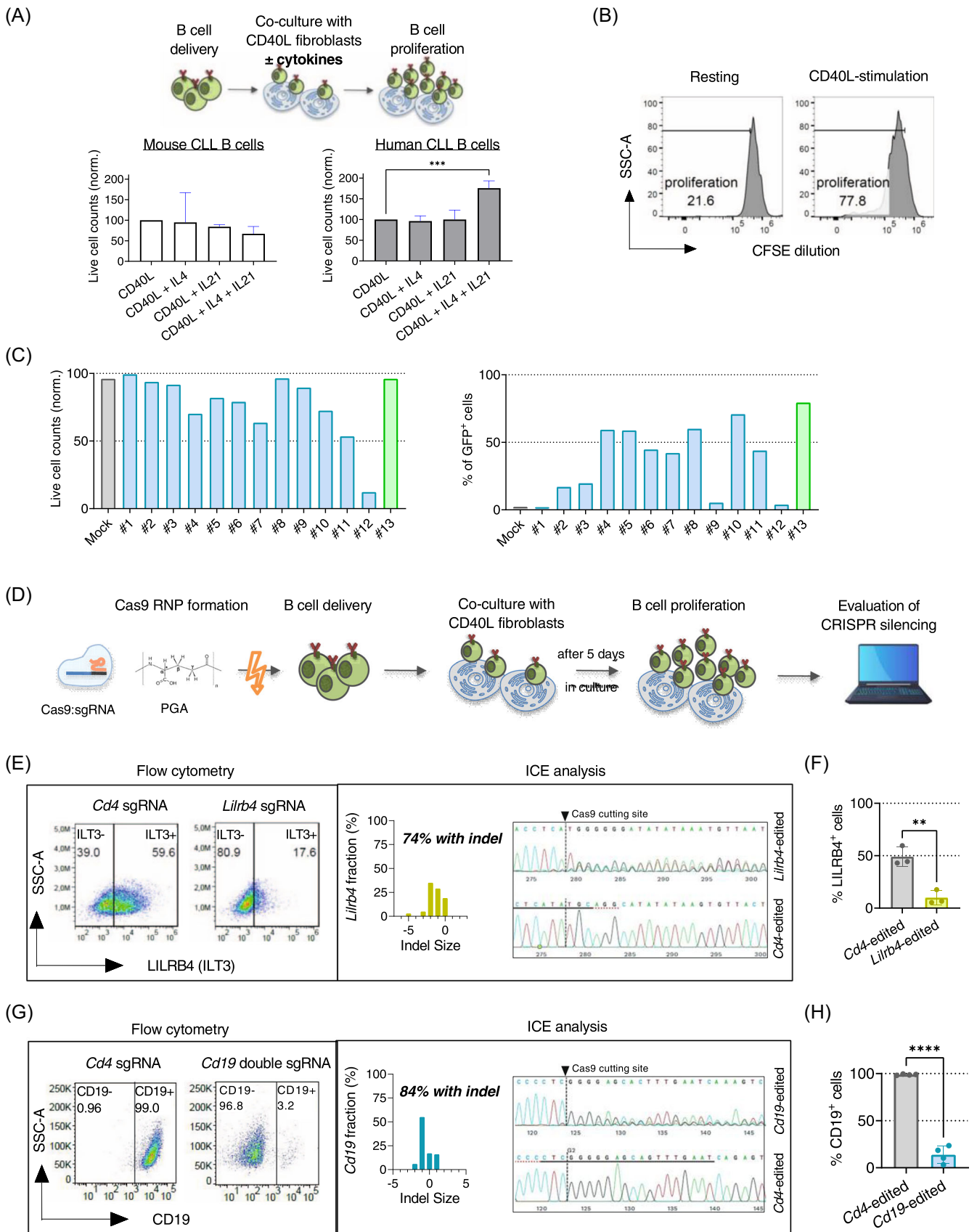
FIGURE 1 (See caption on next page).

FIGURE 1 *In vitro* optimization of culture conditions, electroporation settings, and CRISPR/Cas9 gene editing in TCL1 cells. (A) Total counts of live TCL1 cells (left panel, *n* = 4 biological replicates) and primary human CLL cells (right panel, *n* = 4 donors) cultured at indicated conditions for 5 days. (B) Flow cytometry analysis of TCL1 cell proliferation by CFSE dilution assay. (C) Viability (on the left) and transfection efficiency (on the right) for TCL1 cells electroporated with 3 μg of GFP messenger RNA and cultured overnight. Electroporation setting judged as optimal (#13) is highlighted in green. (D) Workflow of CRISPR/Cas9-based gene editing in TCL1 cells *in vitro*. (E) Representative experiment of silencing of *Lilrb4*, encoding CLL cell surface marker ILT3,[6] 5 days after RNP electroporation. Analysis was done by flow cytometry (left panel) to detect LILRB4/ILT3 protein and *Inference of CRISPR editing* (ICE) analysis (right panel) to evaluate indel frequency in *Lilrb4* gene. (F) Summary of experiments showing *Lilrb4* silencing (*n* = 3) on Day 5 post-RNP electroporation. (G) Representative experiment showing *Cd19* silencing 5 days after RNP electroporation by using a combination of two sgRNAs (optimized in Supporting Information S1: Figure S3). Analysis was done by flow cytometry (left panel) and ICE analysis (right panel). (H) Summary of experiments showing *Cd19* silencing (*n* = 4) after 5 days post-RNP electroporation. In CRISPR-Cas9 experiments (panels F–I), *Cd4*-edited cells were used as control. ********p* > 0.01, ***p < 0.001, ****p < 0.0001.
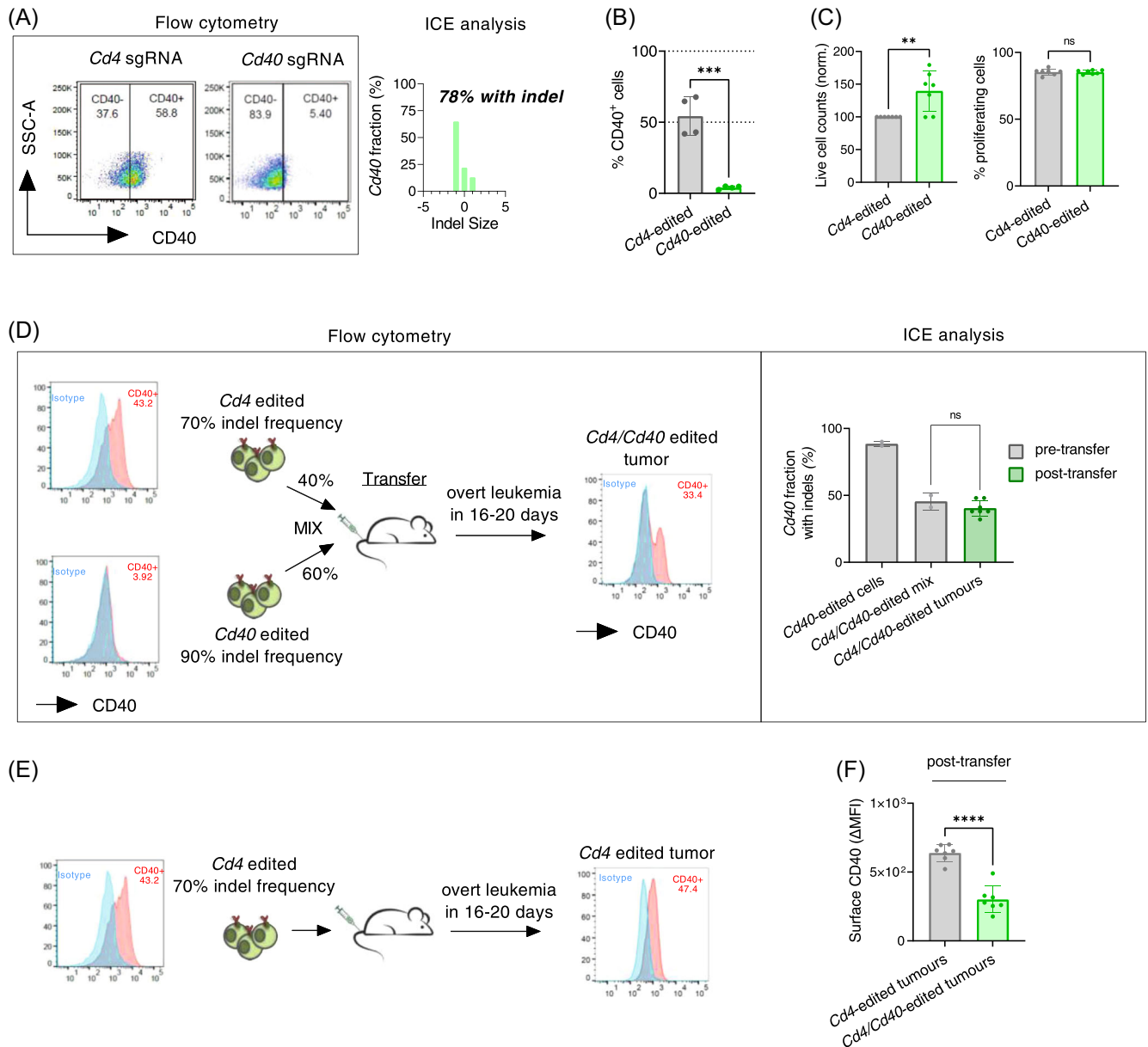
FIGURE 2 Evaluation of *Cd40* silencing for leukemia progression *in vivo*. (A) Representative experiment showing *Cd40* silencing 5 days after RNP electroporation and culturing *in vitro*. Analysis was done by flow cytometry (left panel) and ICE analysis (right panel). (B) Summary of experiments showing efficiency of *Cd40* silencing (*n* = 4 independent experiments) at day 5 post-RNP electroporation. (C) Total counts of live *Cd40*-edited TCL1 cells and proliferation after 5 days in culture (*n* = 7 independent experiments); *Cd4*-edited cells were used as control. (D) *In vivo* competition experiment showing flow cytometry analysis (left panel) and ICE analysis (right panel) of CD40 editing in TCL1 cells before and after injection into C56BL/6 recipients. (E) Flow cytometry analysis of CD40 levels on *Cd4*-silenced TCL1 cells before and after injection into recipients. (F) Flow cytometry comparison of CD40 expression on *Cd4*-silenced and *Cd40*-silenced TCL1 cells before (*n* = 2 independent experiments) and after injection into C56BL/6 recipients (*n* = 7 *Cd4*-edited tumors; *n* = 7 *Cd40*-edited tumors). ns: not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001.

while maximum levels of gene silencing could be achieved by Day 4 of culturing (Figure S4). To expand Cas9/sgRNA-electroporated TCL1 cells *in vivo*, we used intravenous (*i.v.*) route of tumor infection into healthy C57BL/6 recipients[8] allowing for homogeneous tumor engraftment in C57BL/6 recipients at a reduced dose (5×10^6 cells per animal; Supporting Information S1: Figure S5).

In order to apply the newly developed protocol (Supporting Information Methods) to validate a relevant biomarker implicated in CLL pathogenesis, we decided to target the costimulatory receptor CD40, the triggering of which is sufficient to induce TCL1 cell proliferation (Figure 1A). CD40 engagement within CLL proliferative centers is thought to dictate CLL progression *in vivo*[9] and promote resistance to therapies such as the Bcl2 inhibitor venetoclax.[10] We established sgRNA combination that was highly efficient in *Cd40* silencing *in vitro*, while silencing *Cd4* which is not expected to give any phenotype in TCL1 cells as a control (Figure 2A,B). Contrary to what expected, we observed that *Cd40* silencing did not affect CD40-driven TCL1 cell proliferation *in vitro*, suggesting that initial levels of CD40 protein might be sufficient to prime cells for proliferation and that at later time points CD40 signaling becomes dispensable (Figure 2C). We then performed an *in vivo* competition experiment by injecting *Cd40*-edited and *Cd4*-edited TCL1 cells into C57BL/6 recipients (Figure 2D). Resulting tumors maintained constant levels of *Cd40*-silenced tumor population that persisted *in vivo*, which was reflected in the constant frequency of *Cd40* locus indels in bulk tumor population (Figure 2D, right panel) and in a significantly lower CD40 expression compared to control tumors originating from *Cd4*-edited cells (Figure 2E,F). Our findings hence suggested that the loss of CD40 expression did not compromise expansion of leukemic cells *in vivo*. This result goes in line with a recently published observation that wild-type TCL1 cells are able to expand in CD40L$^{-/-}$ hosts.[11] Hence, complementary approaches allowing to test gene function in leukemia progression both by disrupting its expression on tumor cells, or within tumor microenvironment,[11] confidently illustrates that CD40 function may be substituted by other proliferative stimuli within CLL niche *in vivo*.

Our work reports a highly efficient and easily controlled genetic modification protocol for primary leukemic B cells in the TCL1 model. The whole cycle of gene silencing could be completed within 6–8 weeks from the start of sgRNA validation step, allowing to rapidly evaluate gene function in CLL progression. Our protocol routinely achieves over 80% editing efficiency and allows for quality control of CRISPR/Cas9 editing and functional assessment of gene silencing already in the *in vitro* phase. It lowers the cost of editing experiments as the *in vitro* TCL1 cell expansion allows for up to three-fold multiplication of edited cell population and proves effective in engrafting as low as 5×10^6 of edited TCL1 cells per animal via *i.v.* administration. Finally, our method is applicable to multiplexed gene editing *in vitro* and *in vivo* (Supporting Information S1: Figure S6) and could be translated to precise genome editing, wherein Cas9 RNPs are co-electroporated with a DNA template for homology-directed repair,[12–15] and to other B cell types, after appropriate set up of electroporation and expansion conditions.

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AUTHOR CONTRIBUTIONS

Rosita Del Prete and Roberta Drago conceived the experimental design, performed experiments, analysed data and wrote the manuscript; Rosita Del Prete, Roberta Drago, Federica Nardi, Gaia Bartolini, Erika Bellini, Antonella De Rosa, and Silvia Valensin performed and analyzed experiments; Anna Kabanova coordinated the project, supervised experimental design, consulted obtained results, edited the manuscript and provided funding.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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