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LETTER TO THE EDITOR

Cell models with inducible oncogenic translocations allow to evaluate the potential of drugs to favor secondary translocations

Dear Editor,

Chromosomal translocations result from the interchange of genetic material between non-homologous chromosomes. Chromosomal translocations are formed by erroneous repair of double-stranded breaks (DSBs) via non-homologous end joining (NHEJ) [1]. Some genotoxic drugs produce DSBs and thus present a major risk factor for the development of oncogenic chromosomal translocations. The risk factors that interfere with translocation-prone DSB repair, once DSBs are already formed, are obscure, and potential effects of drugs on translocation formation during this step have never been explored.

The study of chromosomal translocations is complicated since naturally occurring translocations are rare, and the localization of breakpoints varies from kilobases to hundreds of kilobases, which complicates their detection. In contrast, when DSBs are generated at precise loci, the translocation can be easily detected by PCR. In the present work, we developed two experimental human B cell-based models to study lymphomagenic t(8;14) *MYC-IGH* and leukemogenic t(8;21) *AML1-ETO* translocations, characteristic for Burkitt's lymphoma (BL) [2] and acute myeloid leukemia (AML), respectively [3]. We used these systems to study pathways and drugs that affect the probability of oncogenic translocations. BL often arises in people living with human immunodeficiency virus (HIV, PLWH) who are treated with combination antiretroviral therapy regimen [4], while chemotherapy is a risk factor for secondary AML [3]; therefore, we tested common antiretroviral and chemotherapeutic drugs for their ability to influence the rate of translocation formation in our systems.

Two experimental systems for the targeted generation of DSBs in either the *AML1* and *ETO* (iAML1-ETO cell line) or *MYC* and *IGH* loci (iMYC-IGH cell line) were created. The models were derived from the RPMI8866 lymphoblastoid cell line with the stable integration of the *Cas9* gene, expressed under the control of a doxycycline (Dox)-inducible promoter and two guide RNA genes (targeting either *AML1-ETO* or *MYC-IGH* loci) (Figure 1A, Supplementary Figure S1). The DSB loci in the *AML1-ETO* model were similar to breakpoints in patients with therapy-related AML and cells treated with etoposide; the DSB loci in the *MYC-IGH* model were similar to breakpoints in patients with sporadic and HIV-induced BL (see Supplementary Methods for further information). The addition of Dox activated Cas9 expression after 4 hours (Figure 1B, Supplementary Figure S2) and stimulated the formation of DSBs in the selected loci (data not shown). The generated t(8;14) or t(8;21) translocations were detectable by qPCR using the primers that surrounded the translocation breakpoint (Figure 1C-D, Supplementary Figure S3). Translocations peaked after 48 hours for iMYC-IGH and 96 hours for iAML1-ETO (data not shown), and these timepoints were selected for further experiments. Without Dox treatment, chromosomal translocations were undetectable.

To get insight into the mechanisms of the translocation generation in our system, we used several inhibitors of DSB

List of abbreviations: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 3TC, Lamivudine; ABC, abacavir; AML, acute myeloid leukemia; a-NHEJ, alternative NHEJ; ANOVA, analysis of variance; ATM, ataxia telangiectasia mutated; AZT, azidothymidine; BL, Burkitt's lymphoma; Cas9, CRISPR associated protein 9; CCR5, C-C Motif Chemokine Receptor 5; c-NHEJ, classical NHEJ; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease9; DNA-Pk, DNA-dependent protein kinase; Dox, doxycycline; DSB, Double-strand break; ENIT, Engineered nuclease-induced translocations; FTC, emtricitabine; HIV, human immunodeficiency virus; IC_{10}, 10% inhibitory concentration; IC_{50}, half-maximal inhibitory concentration; iMYC-IGH, RPMI8866-derived cell line that inducibly express CRISPR/Cas9 and guide RNAs, targeting both *MYC* and *IGH* loci; iAML1-ETO, RPMI8866-derived cell line that inducibly express CRISPR/Cas9 and guide RNAs, targeting both *AML1* and *ETO* loci; HR, homologous recombination; NHEJ, Non-homologous end-joining; PARP, poly(ADP-ribose) polymerase; PCR, Polymerase chain reaction; PLWH, people living with HIV.

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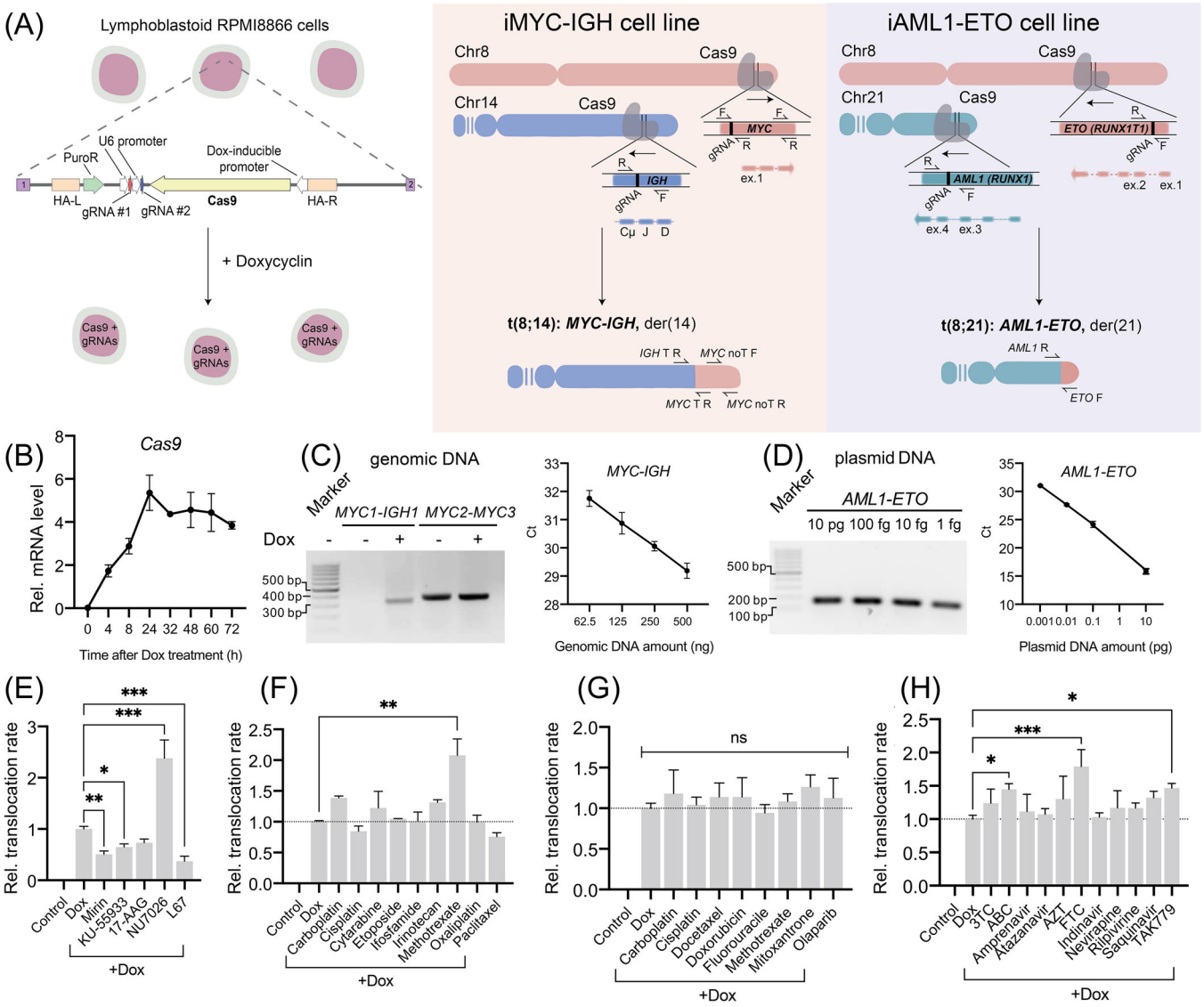

FIGURE 1 RPMI8866-based iMYC-IGH and iAML1-ETO cell models to induce and detect specific chromosomal translocations. (A) General overview of the experimental setup. HA-L and HA-R homology arms were used for the homology-directed genome knock-in of Cas9 and guide RNA genes in the AAVS1 loci on chromosome 19. PuroR, puromycin resistance gene. (B) Kinetics of *Cas9* expression as analyzed by RT-qPCR. The mRNA level was normalized to *GAPDH* expression. (C) Detection of *MYC-IGH* translocations by qPCR: a representative image of agarose gel electrophoresis of PCR products amplified with *MYC1-IGH1* (translocation primers) and *MYC2-MYC3* (primers ~6.8 kbp downstream the breakpoint in *MYC*) from genomic DNA with and without Dox induction (left panel) and the analysis of primer efficiency by plotting the cycle threshold value (Ct) against the dilution of the genomic DNA sample (right panel). (D) Detection of *AML1-ETO* translocations by qPCR: a representative image of agarose gel electrophoresis of *AML1-ETO* PCR products amplified from 10 pg, 100 fg, 10 fg or 1 fg of plasmid DNA (left panel) and the analysis of primer efficiency by plotting the cycle threshold value (Ct) against the dilution of the plasmid DNA sample (right panel). Due to the lower rate of *AML1-ETO* translocations, pUC18-based plasmid containing *AML1-ETO* PCR product was created for the quantification of translocation rate and for the amplification efficiency determination with the standard curve. (E) Effect of DNA repair inhibitors on the *MYC-IGH* translocation rate in iMYC-IGH cells. Cells were simultaneously treated with Dox and the indicated inhibitor or left untreated (control) for 48 h, and translocations were detected by qPCR. Fold changes of t(8;14) translocation rate were calculated relative to that in Dox-treated cells, set as 1. (F) The effect of chemotherapeutic drugs on t(8;21) translocation rate. iAML1-ETO cells were simultaneously treated either with Dox alone to induce the expression of Cas9 and guide RNAs targeting *AML1* and *ETO*, or with chemotherapeutic drugs and Dox, or left untreated (control). Chemotherapeutic drugs were added at a non-lethal 10% inhibitory concentration. At 96 hours later, DNA was collected, and the t(8;21) translocation rate was measured by qPCR. Fold changes of t(8;21) translocation rate were calculated relative to that in Dox-treated cells, set as 1. (G) The effect of chemotherapeutic drugs on t(8;14) translocation rate. iMYC-IGH cells were simultaneously treated either with Dox alone or with chemotherapeutic drugs and Dox, or left untreated (control). At 48 hours later, DNA was collected, and the t(8;14) translocation rate was measured by qPCR. Fold changes of t(8;14) translocation rate were calculated relative to that in Dox-treated cells, set as 1. (H) The effect of antiretroviral drugs on t(8;14) translocation

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repair pathways. iMYC-IGH cells were simultaneously treated with Dox and either Mirin (an MRE11 inhibitor), KU-55933 [(an ataxia telangiectasia mutated (ATM) inhibitor)], 17-N-allylamino-17-demethoxygeldanamycin [17-AAG, an Hsp90 inhibitor that inhibits homologous recombination (HR) repair by destabilizing Rad51], NU7026 [a DNA-dependent protein kinase (DNA-PK) inhibitor, classical NHEJ, (c-NHEJ)], or L67 [an inhibitor of DNA ligase I and III, alternative NHEJ (a-NHEJ)]. Treatment with Mirin, KU-55933 or L67 significantly decreased, whereas treatment with NU7026 significantly increased the *MYC-IGH* translocation rate (Figure 1E). Presumably, the inhibition of MRE11 and ATM, involved in the early steps of DNA damage detection and response, resulted in DSB repair defects [5] and ultimately cell cycle arrest or death, which decreased the overall translocation rates. ATM also promotes the clustering of DSBs into large repair foci [5], which might contribute to DSB proximity and translocations. The inhibition of c-NHEJ increased the translocation rate, while the inhibition of the a-NHEJ pathway decreased the translocation rate, which were in agreement with a previous studies [1]. The increase in the translocation rate when c-NHEJ is abrogated is due to the slow kinetics of DNA repair via a-NHEJ, which permits the free movement of unrepaired DNA ends and increases the chance of meeting their translocation partner in the nuclear space [1].

We next tested whether some chemotherapeutic drugs could influence the translocation formation and thus contribute to the formation of secondary neoplasms, e.g., therapy-related AML. We used drugs of different classes: platinum-based antineoplastics (carboplatin, cisplatin, oxaliplatin), topoisomerase inhibitors (doxorubicin, etoposide, irinotecan, mitoxantrone), antimetabolites (cytarabine, fluorouracil, methotrexate), poly(ADP-ribose) polymerase (PARP) inhibitor (olaparib), an alkylating agent (ifosfamide), and cytoskeletal drugs (docetaxel, paclitaxel). To exclude the effects related to cytotoxicity and cell death, we chose the non-lethal 10% inhibitory concentration (IC_{10}) of the above drugs (Supplementary Table S3, Supplementary Figure S4). iAML1-ETO cells were simultaneously treated with Dox and chemotherapeutic drugs, and the level of t(8;21) translocations was measured and compared to those in Dox-treated cells. We found that the addition of methotrexate increased more than two-fold the rate of *AML1-ETO* translocations (Figure 1F). No significant differences were found for other drugs, which was further confirmed in iMYC-IGH cells with five other chemotherapeutic drugs used at IC_{10}. No significant differences were found between the translocation rate in Dox-treated and Dox+drug-treated iMYC-IGH cells (Figure 1G). These results indicated that in the iMYC-IGH model, chemotherapeutic drugs did not interfere with the DSB repair once DSBs were formed. Thus, methotrexate use can increase the risk of secondary therapy-related AML, and patients treated with methotrexate should be monitored for the development of AML. Noteworthy, the development of therapy-related myelodysplastic syndrome/AML with t(8;21) and t(3;21) translocations was described in patients following low-dose treatment with methotrexate for rheumatoid arthritis [6].

We next tested whether antiretroviral drugs could influence the level of *MYC-IGH* translocations since BL is a common neoplasm in PLWH. We used drugs from different classes, such as nucleoside reverse transcriptase inhibitors (NRTIs) [abacavir (ABC), azidothymidine (AZT), emtricitabine (FTC), lamivudine (3TC)], non-NRTIs (nevirapine, rilpivirine), protease inhibitors (amprenavir, atazanavir, indinavir, saquinavir), and C-C motif chemokine receptor 5 (CCR5) antagonist (TAK779). The drugs were added simultaneously with Dox to the iMYC-IGH cell medium at the reported IC_{50} for virus inhibition. The chosen concentrations were not toxic for the cells (Supplementary Figure S4C). We found that two NRTIs, ABC and FTC, and CCR5 antagonist TAK779 significantly increased the rate of *MYC-IGH* translocations (Figure 1H). NTRIs are known to be incorporated into nuclear DNA by certain DNA polymerases during DNA repair, and they can act as chain terminators and directly inhibit cellular DNA polymerases by binding to their catalytic site [7]. Compromised DNA damage response may delay the classical repair machinery and engage translocation-prone a-NHEJ. Furthermore, mitochondrial toxicity of NRTIs could affect nuclear

rate. iMYC-IGH cells were simultaneously treated either with Dox alone or with antiretroviral drugs and Dox, or left untreated (control). At 48 hours later, DNA was collected, and the t(8;14) translocation rate was measured by qPCR. Fold changes of t(8;14) translocation rate were calculated relative to that in Dox-treated cells, set as 1. All data are plotted as mean ± SEM. ns, non-significant; *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared by ANOVA, Dunnett's post-test relative to Dox-treated cells. The experiments were carried out in at least three biological replicates. Abbreviations: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 3TC, Lamivudine; ABC, abacavir; AML, acute myeloid leukemia; AZT, azidothymidine; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9; Dox, doxycycline; FTC, emtricitabine; gRNA, guide RNA; HA, homology arms; iMYC-IGH, RPMI8866-derived cell line that inducibly express CRISPR/Cas9 and guide RNAs, targeting both *MYC* and *IGH* loci; iAML1-ETO, RPMI8866-derived cell line that inducibly express CRISPR/Cas9 and guide RNAs, targeting both *AML1* and *ETO* loci; kbp, kilobase pairs; PCR, Polymerase chain reaction; PuroR, puromycin resistance gene.

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DNA integrity through reactive oxygen species production and imbalanced deoxynucleoside triphosphate pools [8]. CCR5 governs DNA damage repair (HR and single-strand annealing); consequently, CCR5 inhibitors were shown to sensitize cells to DNA-damaging agents [9]. Defects in HR and c-NHEJ repair mechanisms can result in chromosomal translocations [1]. A phase II clinical study of vicriviroc, a CCR5 antagonist, showed that vicriviroc treatment was associated with an increased risk of developing lymphomas [10]. Vicriviroc was not approved for HIV treatment.

To conclude, we developed CRISPR/Cas9-based cell models with inducible *AML1-ETO* and *MYC-IGH* translocations to evaluate the potential of drugs to favor secondary translocations. In our screen, we identified four drugs, methotrexate, ABC, FTC and TAK779, that increased the rate of chromosomal translocations.

DECLARATIONS

AUTHOR CONTRIBUTIONS

A.S. and N.L. performed the following experiments: cell line creation and characterization, experimental setup, translocation rate analysis, survival analysis, RT-qPCR, western blotting, analyzed data and wrote the paper; D.S. and V.P. performed the following experiments: translocation rate and survival analysis and contributed new reagents; V.V., T.T., D.G. and Y.K. performed the following experiments: translocation rate analysis, RT-qPCR, western blotting; M.S. and M.R. analyzed data; Y.V. designed research, analyzed data and wrote the paper.

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COMPETING INTERESTS

The authors declare that they have no competing interests

AVAILABILITY OF DATA AND MATERIALS

Methods and materials are available in the supplementary file. All data generated or analyzed during this study are included in this published article and its supplementary information files.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

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