A Fibrinogen Alpha Fragment Mitigates Chemotherapy-Induced MLL Rearrangements

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Rearrangements in the Mixed Lineage Leukemia breakpoint cluster region (MLLbc) are frequently involved in therapy-induced leukemia, a severe side effect of anti-cancer therapies. Previous work unraveled Endonuclease G as the critical nuclease causing initial breakage in the MLLbc in response to different types of chemotherapeutic treatment. To identify peptides protecting against therapy-induced leukemia, we screened a hemofiltrate-derived peptide library by use of an enhanced green fluorescent protein (EGFP)-based chromosomal reporter of MLLbc rearrangements. Chromatographic purification of one active fraction and subsequent mass spectrometry allowed to isolate a C-terminal 27-mer of fibrinogen α encompassing amino acids 603 to 629. The chemically synthesized peptide, termed Fa27, inhibited MLLbc rearrangements in immortalized hematopoietic cells following treatment with the cytostatics etoposide or doxorubicin. We also provide evidence for protection of primary human hematopoietic stem and progenitor cells from therapy-induced MLLbc breakage. Of note, fibrinogen has been described to activate toll-like receptor 4 (TLR4). Dissecting the Fa27 mode-of-action revealed association of the peptide with TLR4 in an antagonistic fashion affecting downstream NFκB signaling and pro-inflammatory cytokine production. In conclusion, we identified a hemofiltrate-derived peptide inhibitor of the genome destabilizing events causing secondary leukemia in patients undergoing chemotherapy.

Keywords: bioactive peptide, doxorubicin, Endonuclease G, hematopoietic stem and progenitor cells, inflammatory signaling, mixed lineage leukemia

INTRODUCTION

Chromosomal translocations, inversions, amplifications and complex rearrangements at the 11q human genomic locus encoding the lysine methyl transferase 2 A gene (KMT2A), also known as mixed lineage leukemia gene (MLL), are a hallmark of acute lymphoblastic leukemia (ALL) in infants and of therapy-induced acute myeloid leukemia/myelodysplastic syndrome (AML/MDS)
adults (1–3). MLL abnormalities were reported to be detectable in up to 85% of infant ALL and approximately one third of therapy-induced AML cases (2, 4). Underscoring their impact, these chromosomal changes correlate with poor prognosis, resistance to treatment and reduced overall survival (5, 6). Infant ALL cases have causally been linked with exogenous sources such as exposure to carcinogenic dietary components during fetal hematopoiesis (7–9). Therapy-induced leukemias have been observed after treatments with various cytostatics ranging from nucleoside analogs to alkylating agents, with a particularly short latency period after chemotherapies with anti-topoisomerase II inhibitory compounds like the epipodphyllotoxin etoposide or DNA intercalating drugs like the anthracycline doxorubicin (10–14).

Providing further support for a mechanistic connection between infant ALL and therapy-induced AML, the vast majority of aberrations fall within a 0.4 kb breakpoint cluster region hotspot (MLLbcr) that is distinct from the cluster of MLL rearrangements in adult de novo ALL [reviewed in (2, 15)]. The MLLbcr sequence has been predicted to adopt complex secondary structures with hairpins that are known to be highly enriched at translocation breakpoints in human cancer genomes (2, 16). Such non-B DNA structures represent obstacles to the transcription and DNA replication machinery and are therefore subject to incisions by nucleaseases. MLLbcr rearrangements can be explained by error-prone repair attempts in apoptosis-escaping cells and can be recapitulated ex vivo in cultured lymphocytes as well as hematopoietic stem and progenitor cells (HSPCs) after exposure to nutritional risk factors or cytostatic drugs (8, 9, 17–19).

In our previous work we unraveled a therapy-induced replication stress-signaling pathway, which triggers nuclear accumulation of Endonuclease G (EndoG) and specific MLLbcr cleavage, whereby EndoG is recruited by the base excision repair machinery in an attempt to resolve stalled replication forks. Importantly, we showed that in cycling cells MLLbcr cleavage by EndoG is necessary for misrepair resulting in MLLbcr rearrangements to occur (19, 20). Of note, HSPCs, the leukemia cells-of-origin, are forced to enter the cell cycle upon stress stimuli triggering bcr cleavage and consequently treatment-induced AML in patients treated with various types of cytostatics (2).

Etoposide and doxorubicin are widely applied in anti-cancer treatments showing efficacy against solid tumors, leukemias and lymphomas, including bladder, lung, breast and thyroid cancers, Hodgkin’s lymphoma, multiple myeloma and others (11, 21). However, their clinical use and their dosage are limited by adverse effects, mostly cardio- and hematological toxicities as well as treatment-induced leukemia. Etoposide is a topoisomerase II inhibitor known to cause MLL bcr breakage (22). In our previous work we found EndoG to be involved in MLLbcr cleavage after etoposide treatment (20). Anthracyclins like doxorubicin exert multiple biological activities inducing DNA damage via DNA intercalation and generation of reactive oxygen species (ROS) and have as well been linked with MLL rearrangements in therapy-induced leukemia (23–25). In search of cytoprotective compounds, Jang and colleagues (26) screened and found two small molecule EndoG inhibitors. However, indiscriminate loss of all EndoG functions is not desirable given its importance in the homeostasis of mitochondria (27), an assumption which is supported by the cardiotoxic effects observed in knockout mice (28).

Given that the human peptidome is a large unexplored source of bioactive peptides with unknown functions that may be involved in MLLbcr rearrangements, we chose a screening approach to identify endogenous, hemofiltrate-derived peptides that mitigate these rearrangements during etoposide and doxorubicin treatments. Employing EGFP-based reporter cells we discovered such a peptide, namely a C-terminal 27-mer cleavage product of fibrinogen α, and discovered that it antagonizes toll-like receptor 4 (TLR4) - NFκB signaling. Our work provides evidence that inflammatory signaling plays a key modulatory role in one of the most severe side effects of cytostatic treatments. We further provide proof-of-concept that a peptide can block chemotherapy-induced localization of EndoG in the nucleus and therefore MLLbcr rearrangements by manipulating this pathway from the cellular surface.

**MATERIAL AND METHODS**

**Cell Culture**

Human WTK1 (29) and K562 (#ATCC CCL 243, American Type Cell Culture Collection) as well as the reporter cell line derivatives WTK1MLL and K562MLL, both stably transfected with the DNA recombination substrate pHr-EGFP/3’EGFP-MLLbcr.fwd (30), were cultured in RPMI 1640 (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and 5% L-Glutamine (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA). For serum-free experiments AIM V™ Medium (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. The HEK-Blue™ hTLR4 cells obtained by co-transfection with TLR4, MD-2 and CD14 co-receptor genes and an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene (InvivoGen, San Diego, California, USA) were cultured in DMEM without glucose, glutamine and phenol red (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), which was supplemented with 4.5 g/l glucose (Sigma-Aldrich/Merck, St. Louis, Missouri, USA), 2 mM L-glutamine (Gibco/Thermo Fisher Scientific), 10% fetal bovine serum (Biochorm, Berlin, Germany), 1x penicillin-streptomycin (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), 100 µg/ml Normicin™ (InvivoGen, San Diego, California, USA) and 1x HEK-Blue™ selection (InvivoGen, San Diego, California, USA). HeLa cells (ECACC, UK, London, 93021013) were cultured in DMEM (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) including 10% FBS (Sigma-Aldrich/Merck, St. Louis, Missouri, USA), 1% sodium pyruvate (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1% non-essential amino acids (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts,
Human hemofiltrate was collected and analyzed considering ethical vote #91/17 (University of Ulm) and with the patient’s written informed consent prior to inclusion in the study. Umbilical cord blood samples were collected in the Department of Obstetrics and Gynecology of Ulm University and informed consent was obtained from mothers within 24 h after birth. Blood sample collections were approved by the local advisory board (approval #155/13, University Ulm). Hematopoietic stem and progenitor cells (HSPCs) were isolated by Melanie Rall-Scharpf and Heike Schreier.

Culturing was performed in a humid 5% CO₂ incubator at 37°C. All cell lines were tested negative for mycoplasma by PCR.

**Treatment and Measurement of MLLbcr Rearrangements via FACS Analysis**

K562MLL or WTK1MLL cells (1-2 x10⁶ cells in a final volume of 2 ml) were seeded in RPMI 1640 or AIM V™ medium and subjected to serial treatments according to the experimental procedure described in the legend of each figure. In the experiments with continuous treatment, a 4 h pre-treatment with Fxx27 was followed by an exposure to peptide plus 10 µM etoposide for 72 h (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) or doxorubicin (University Hospital Ulm, Germany) at the approximate IC50 dose (WTK1MLL: 0.5 µM). In the experiments with release of WTK1MLL cells from drug treatments, the 4 h incubation with Fxx27 peptide was followed by another 4 h incubation period with peptide plus 0.5 µM doxorubicin. Subsequently, cells were washed with 2 ml of PBS and seeded in 2 ml of fresh AIM V™ medium without peptide and cytostatic drug followed by 72 h of cultivation. For the determination of recombination frequencies adjacent to the MLLbcr (MLLbcr rearrangements) the medium was removed and the cells were resuspended in PBS with 0.2% EDTA. The fractions of EGFP-positive cells among living cells were measured flow cytometrically by use of a FACSCalibur™ (BD Biosciences, San Jose, California, USA), whereby living cells were selected by the FSC/SSC gate and green fluorescent cells therein detected in the diagonal FL1/FL2 dot plot gate (31, 32). Autoluminescence of doxorubicin led to a fluorescence signal shift of the cells during FACS analysis requiring gate adjustment by a treated control without EGFP-reporter. To correct for inter-experimental variation mean recombination frequencies in peptide-free etoposide/doxorubicin controls were set to 100% in each experiment and relative percentages calculated for each single value. Peptide concentrations were chosen according to concentration-dependent responses of the different cell types during recombination measurements. Thus, WTK1MLL cells were exposed to 1 mg/ml Fxx27 for etoposide and 10 µg/ml Fxx27 for doxorubicin treatments to reach down-regulation of MLLbcr rearrangements by at least 40%.

For the experiments engaging disulfiram (Sigma-Aldrich/ Merck, St. Louis, Missouri, USA) or N-acetyl cysteine (NAC) (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) additional pre-incubation times of 5 h for disulfiram or 1 h for NAC were added to the protocol, i.e. before the peptide incubation period, whereby disulfiram or NAC were kept in the media as long as peptide and cytostatic treatments took place.

**Human Hemofiltrate-Derived Peptide Screen**

A peptide library was generated starting from hemofiltrate of patients suffering from chronic renal failure as previously described (33). Briefly, blood was filtered across a membrane with a cut-off of 20-30 kDa. Peptides in the filtrate were separated by cation-exchange chromatography resulting in eight eluates which were then separated by reverse phase HPLC into 48 fractions each (34). First round screening of the resulting 384 fractions was performed in K562MLL cells with a chromosomally integrated EGFP-based reporter construct for MLLbcr rearrangements (30). Cells were seeded in 2 ml culture medium and pre-treated for 4 h with 90 µl of each fraction corresponding to 5 l of hemofiltrate. Subsequently, the chemotherapeutic etoposide (10 µM) was included and cells cultivated for 72 h until recombination measurements by FACS. Fractions inducing statistically significant decreases in mean MLLbcr recombination frequencies were subjected to second round screening after rechromatography by analytical reversed phase chromatography. Second round screening of fraction E8F08 revealed fraction 11 with the most pronounced downregulation of etoposide-induced recombination and was subjected to MALDI mass spectrometry analysis and Edman Sequencing. For MALDI-MS samples were re-suspended in a MALDI matrix solution containing alpha-cyano-4-hydroxy cinnamic acid (HCCA, 8 mg/ml, Fluka, Morristown, USA) with 6-desoxy-L-galactose additive in 50/49/1 (v/v) 0.2% TFA/acetonitrile/acetone. Then, 0.5 µl of the sample/matrix mixture were spotted on a target and spectra were acquired in linear mode (Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF). Sequencing was carried out by conventional Edman degradation detecting the phenyl isothiocyanate labeled amino acids (33, 34).

**Peptide Synthesis and Purification**

The peptides were synthesized using standardized Fmoc chemistry by solid-phase peptide synthesis (SPPS) on an automated peptide synthesizer (Liberty blue, CEM, Kamp-Lintfort, Germany). An amino acid with a protecting group was added to the first amino acid, which was linked to the resin. After activation the subsequent amino acid was coupled to grow the peptide chain in cycles. Finally, the full-length peptide was removed from all protecting groups and the solid-phase resin. The peptide was purified with a HPLC-1100 and characterized via mass spectrometry. The crude peptide was stored at 4°C and after solubilization at -20°C. The sequences of the used peptides were Fxx27 (MADEAGSEADHEG THSTKRGHAKSRPV), Scramble 1 (LASCEAYIKGRNVHTDP GLGASVLQAF), Scramble 2 (RSDDEHASKHVTPEEA GRMSATAGSK), Fxx27-Flag (MADEAGSEADHEG THSTKRGHAKSRPVVSXSXDYYMKDDDK) and Flag-Fxx27 (DYKDDDQGSSGGSMADAEAGSEADHEGTHSTKRGH AKSRPV). 10 µg/ml Fxx27, Scramble 1 and Scramble 2...
correspond to 3.5 μM, 15 μg/ml Fz27-Flag and Flag-Fz27 correspond to 3.5 μM.

**Cell Cycle Analysis**

Cell cycle analysis was performed as detailed in the Supplement.

**DNA Double-Strand Break (DSB) Repair Analysis via FACS**

WTK1 or WTK1MLL cells were seeded at a density of 4 x 10^6 in AIM V™ medium and treated with 1 mg/ml Fo27 for 4 h. After the pre-incubation period cells were washed with RPMI 1640 Medium, with neither glutamine nor phenol red (Gibco, Thermo Fisher Scientific) and resuspended in 400 µl of this medium. For comparative analysis of extrachromosomal DSB repair activities 10 µg of pCMV-I-SceI plasmid expressing the rare-cutting meganuclease I-SceI and 10 µg of the plasmid EJ-EGFP for determination of MMEJ or of HR-EGFP/3′EndoG for homologous repair (35) were added to the WTK1 cells. For analysis of chromosomal homologous repair, the stable reporter WTK1MLL cells were electroporated with 10 µg plasmid expressing I-SceI only. Mixtures of plasmids and cells were transferred in a Gene Pulser®/Micropuls® electroporation cuvette 4 mm (Bio-Rad Laboratories, Hercules, California, USA) and pulsed at 200 V and 1050 µF with the Gene Pulser Xcell™ electroporation system (Bio-Rad Laboratories, Hercules, California, USA). The cells were added to AIM V™ Medium and the Fo27 concentration adjusted. For the determination of DSB repair frequencies the medium was removed, the cells were resuspended in PBS/0.2% EDTA and the fraction of green fluorescent cells among living cells (FSC/SSC gate) determined with a FACSCalibur™ (BD Biosciences, San Jose, California, USA) using the diagonal gating method in the FL-1/FL-2 dot plot (35). Living EGFP-positive cell fractions were normalized to the transfection efficiencies to calculate the DSB repair frequency. Mean recombination frequencies in peptide-free controls were set to 100% in each experiment and relative percentages calculated.

**Bulk and Single Molecule Imaging of Fo27**

One day before the measurement, HeLa cells were grown at 37°C and 5% CO₂ in DMEM medium (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) including 10% charcoal-stripped FBS (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1% sodium pyruvate (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1% non-essential amino acids (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1% Glu-tMAX (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 35 mm glass bottom µ-dishes (Ibidi, Gräfelfing, Germany).

Before the measurement, we added TAMRA-Fo27 (Pepscan, RC Lelystad, The Netherlands) in a final concentration of 0.27 μM in OptiMEM (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated the cells for 4 h at 37°C and 5% CO₂. Afterwards, we imaged the cells on a custom built spinning disk microscope (36) using a 532 nm laser to visualize TAMRA-Fo27.

For single molecule imaging of TAMRA-Fo27, we followed the same procedure, but grew cells on Delta-T glass bottom dishes (Bioptechs Inc., Butler, Pennsylvania, USA). The final concentration of TAMRA-Fo27 was 2.7 nM. We imaged single TAMRA-Fo27 molecules using HILO microscopy (36) with a 561 nm laser at 1 kW/cm² and 10 ms acquisition time.

**Immunofluorescence Microscopy**

After a 4 h treatment with Fo27 and 4 h with Fo27 plus cytostatic at the indicated concentrations the cells were washed with PBS and spun on microscopic slides coated with Poly-L-Lysine (Sigma-Aldrich/Merck, St. Louis, Missouri, USA). Then, they were pre-extracted with 0.2% Triton/PBS for 30 s and washed for 5 min with PBS. For fixation the slides were incubated for 10 min in 3.7% formaldehyde and washed with PBS three times. Subsequently, cells were permeabilized with 0.5% Triton/PBS for 12 min and PBS washed three times followed by blocking with 5% goat serum/PBS for 1 h. For immunostainings the primary antibodies directed against γH2AX (Merck Millipore, Burlington, Massachusetts, USA 05-636) or EndoG (Santa Cruz Biotechnology, Dallas Texas, USA, sc-365359) were applied 1:1000 in 5% goat serum/PBS for 1 h at 37°C. Incubation with the secondary antibody Alexa Fluor® 488 (Thermo Fisher Scientific, Waltham, Massachusetts, USA, A11001) at 1:1000 in 5% goat serum/PBS took place for 45-60 min at 37°C followed by washing and mounting with VectaShield Mounting Medium for Fluorescence with Dapi (Vector Laboratories, Burlingame, California, USA) and sealed under cover slips. The slides were subjected to high content imaging with a BZ-9000 microscope (Keyence, Neu-Isenburg, Germany), objective 100x/1.45 oil (Nikon, Tokio, Japan) and automated analysis of immunostained foci in DAPI-stained nuclei with BZ-H Analyzer software (Keyence, Neu-Isenburg, Germany). To ensure detection of specific signals negative controls without primary antibodies were included and the same exposure times as well as intensity and minimal focus size thresholds kept throughout each experimental set.

**Proximity-Ligation-Assay (PLA)**

HEK-Blue™ HTLR4 cells were seeded on cover slips coated with sterile Poly-L-Lysine (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) for 72 h. Then, the cells were washed with PBS and treated for 2 h with H₂O, Flag-Fo27 or Fo27-Flag in two different concentrations 15.09 µg/ml and 150.9 µg/ml corresponding to 10 µg/ml Fo27 and 100 µg/ml Fo27, i.e. 3.5 µM and 35 µM peptides, respectively. After treatment the cells were washed for 5 min with PBS and fixed for 10 min in 3.7% formaldehyde. The PLA staining was performed following the manufacturer’s instructions (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) using Duolink® In Situ Detection Reagent Orange, Duolink® In Situ PLA Probe Anti-Rabbit PLUS, Duolink® In Situ PLA Probe Anti-Mouse MINUS and Duolink® In Situ Mounting Medium with DAPI. The primary antibodies TLR4 (Santa Cruz Biotechnology, Dallas Texas, USA, sc-293072), Anti-DDDDK tag/Flag tag (Abcam, Cambridge, UK, ab205606) and anti-PLA2G2D (Abcam, Cambridge, UK, ab24182) were incubated overnight at 4°C. Image analysis was performed as
described for immunofluorescence microscopy. To ensure detection of specific signals negative controls with TLR4 or Flag antibody only or PLA without any antibody were performed. Minimal focus size thresholds and foci size were set independently for each experiment and kept throughout each experimental set.

**Chromatin Immunoprecipitation (ChIP)**

After a 4 h treatment with Fo27 and 4 h with Fo27 plus cytostatic the cells were fixed for 10 min in 1% formaldehyde in the medium on a shaker. Then, glycine was added to the medium to a final concentration of 138 mM and shaking continued for 5 min. Subsequently, cells were centrifuged at 4°C. All following steps were executed on ice or at 4°C using ice cold buffers. The cells were washed with PBS, centrifuged, resuspended with PBS, transferred to a 1.5 ml reaction tube, re-centrifuged, the supernatant removed and the cells shock-froze in liquid nitrogen and stored at -80°C for further use. Cell pellets were dispensed in 1 ml cell lysis buffer (5.0 mM Pipes, pH 8.0; 85 mM KCl; 0.5% NP-40) with 0.5 mM PMSF and 0.5% PIC. For pre-clearing, the supernatant was removed and the remaining cell suspensions were thawed on ice, centrifuged at 14,000 rpm for 10 min and sonicated 6 times for 10 s with intermittent 1 min breaks. The resulting suspension was frozen in liquid nitrogen and stored at -80°C for further use. Aliquots (50 µl) taken before freezing were diluted fourfold in pure water, NaCl added to a final concentration of 250 mM and samples incubated overnight at 65°C. Thereafter, 0.05 µg/µl RNaseA (Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added and the sample incubated for 15 min at 37°C followed by 0.025 µg/µl Proteinase K (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) treatment for 90 min at 42°C. DNA and proteins were separated with phenol-chloroform extraction and the DNA precipitated with 0.2 M sodium acetate (pH 5.2) and 62.5% ethanol overnight at -20°C. DNA pellets were dried and resuspended in 30 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1% SDS), incubated for 10 min and sonicated 6 times for 10 s with intermittent 1 min breaks. The resulting suspension was frozen in liquid nitrogen and stored at -80°C for further use. Aliquots (50 µl) taken before freezing were diluted fourfold in pure water, NaCl added to a final concentration of 250 mM and samples incubated overnight at 65°C. Thereafter, 0.05 µg/µl RNaseA (Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added and the sample incubated for 15 min at 37°C followed by 0.025 µg/µl Proteinase K (Sigma-Aldrich/Merck, St. Louis, Missouri, USA) treatment for 90 min at 42°C. DNA and proteins were separated with phenol-chloroform extraction and the DNA precipitated with 0.2 M sodium acetate (pH 5.2) and 62.5% ethanol overnight at -20°C. DNA pellets were dried and resuspended in 30 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). These samples were used for input DNA assessment. The remaining cell suspensions were thawed on ice, centrifuged and volumes containing 2-20 µg DNA diluted in a total volume of 300 µl dilution buffer (16.7 mM Tris-HCl, pH 8.0; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS; 1.1% Triton X-100 including 0.5 mM PMSF and 0.5% PIC). For pre-clearing protein G sepharose-salmon sperm DNA beads were added, i.e. Protein G Sepharose™ 4 Fast Flow (GE Healthcare, Chicago, Illinois, USA) in 50 µl TE-buffer supplemented with 1 mg/ml BSA and 0.05% sodium azide including 0.4 mg/ml Sheared Salmon Sperm DNA (Ambion® by life technologies™/Thermo Fisher Scientific, Waltham, Massachusetts, USA). Thereafter samples were rotated for 1-2 h. Following centrifugation, pre-cleared supernatants were transferred to new reaction tubes and incubated together with the antibody of interest: γH2AX, Merck Millipore, Burlington, Massachusetts, USA, 05-636; EndoG, Santa Cruz Biotechnology, Dallas Texas, USA, sc-365339; mouse IgG, Santa Cruz Biotechnology, Dallas Texas, USA, sc-2025. After rotation overnight 50 µl of protein G-agarose-salmon sperm DNA beads were added and rotation continued for 2 h. Samples were centrifuged and bead pellets washed four times at room temperature by 10 min rotation and centrifugation with high-salt wash buffer (50 mM HEPES, pH 7.9; 500 mM NaCl; 1.0 mM EDTA; 0.1% SDS; 1.0% Triton X-100; 0.1% deoxycholate) and twice with TE. Beads were resuspended in 300 µl elution buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1.0% SDS) and incubated 2 h at 55°C with 0.0625 µg/µl proteinase K. Afterwards, samples were incubated overnight at 65°C, then centrifuged and supernatants transferred into new reaction tubes. The DNA was isolated as described before and amplified by PCR. The PCR products were separated by agarose gel electrophoresis (2.5%) and visualized by SYBR Save DNA gel stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The primer (Biomers.net, Ulm, Germany) sequences were as follows: For amplification of the HR-EGFP/MLLbcr fragment fwd primer 5’-ACCACACTACAGCAGAACACC-3’ and rev primer 5’-ATACGAAAACGTGTGAAATCGTGC-3’; for HR-EGFP/MLLbcr nested PCR fwd primer 5’-ACAACACTACCTGGACCC-3’ and rev primer 5’-GCTTGATATCGAATCTGCAGGC-3’; for GAPDH fwd primer 5’-CCCAACTTTCGGCGCCTC-3’ and rev primer 5’-CAGGCCCGCTTGATCACTG-3’.

**qRT-PCR**

WTK1MLL cells were treated as described for the analysis of MLLbcr rearrangements via FACS. Total RNA isolation was done with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Reverse transcription was performed using the QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany) following the instructions for 1 µg RNA. For qPCR 3.3 µl cDNA (1:20 diluted) were mixed with 5 µl SensiFAST Probe LO-ROX mix (2x) from the SensiFAST Probe LO-ROX Ki (Bioline, London, UK), 0.5 µl sensor primer and 1.2 µl H2O. The PCR was performed on the qTower (Analytik Jena, Jena, Germany) by using the program 2 min 95°C and 40 cycles of 10 s 95°C and 20 s of 60°C. Controls were ACTB VIC (Thermo Fisher Scientific, Waltham, Massachusetts, USA), HSP90 VIC (Thermo Fisher Scientific, Waltham, Massachusetts, USA), YWHAZ VIC (Thermo Fisher Scientific, Waltham, Massachusetts, USA), ACTB HEX (Bio-Rad Laboratories, Hercules, California, USA) and YWHAZ HEX (Bio-Rad Laboratories, Hercules, California, USA). The genes of interest were analyzed with IFN-gamma Fam, IL-6 Fam, IL-8 Fam and TLR4 Fam (Bio-Rad Laboratories, Hercules, California, USA).

**HEK-Blue™-Assay**

For the HEK-Blue™-Assay the HEK-Blue™ hTLR4 cells were detached and resuspended in HEK-Blue™ Detection (InvivoGen, San Diego, California, USA) at a density of 140000 cells/ml. The peptides were diluted in PBS and 15 µl of the solutions added to the wells to reach the indicated
concentrations in the final volume of 200 µl. Subsequently, 180 µl of the cell suspensions were added for 2 h. Thereafter, cells were treated with final concentrations in 200 µl of 10 ng/ml LPS (InvivoGen, San Diego, California, USA) for 10 h or 0.16 µM doxorubicin (IC80 for 24 h treatment) for 4 h diluted in a volume of 5 µl. Then, doxorubicin treated cells were released into fresh medium. For the release, the cells were centrifuged, the supernatant removed and cells resuspended in 100 µl 1xPBS. After a second centrifugation step the supernatant was removed and 20 µl 1xPBS and 180 µl HEK-Blue™ Detection added for 72 h. Finally, optical densities were measured at 655 nm.

Breakage PCR of the MLLbcr in HSPCs

Human stem and progenitor cells (HSPCs) were isolated from cord blood essentially as described in Kraft et al. (18). Cells were thawed at 37°C. One cell vial equivalent to RPMI 1640 (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) with 10% fetal bovine serum (Biochrom, Berlin, Germany) was slowly dropped to the cell suspension. After an incubation time of 1 min another volume equivalent to the cell mixture with medium was added to the cells by slowly dropping. This step was repeated three times. After centrifugation, the cells were suspended in StemSpan™ SFEM (Stemcell Technologies, Vancouver, Canada) with 1% penicillin-streptomycin (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1% StemSpan™ CC100 (Stemcell Technologies, Vancouver, Canada). HSPCs were cultured at a density of 0.75x10^6 cells/ml for 72 h. For the experiment the cells were seeded in fresh culture medium, treated for 2 h with 10 µg/ml F27 followed by 4 h treatment with the IC90 dose of doxorubicin (0.072 µM) pre-established in WTK1 cells. Genomic DNA was isolated with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following previously established and demonstrated to induce a one order of magnitude increase of MLLbcr rearrangements in K562MLL and WTK1MLL reporter cells derived from human erythroleukemia and human lymphoblastoid cells, respectively (30). Initially, two values (recombination frequencies determined by FACS) were obtained with each fraction. The 45 fractions that showed significant differences in recombination frequency by χ² test were used to obtain additional measurements enabling more strict statistical analysis. This identified three fractions inducing statistically significant changes, namely E5F40 as well as E6F16 with stimulatory and, most interestingly, E8F08 with inhibitory effects protecting against MLLbcr rearrangements (Figure 1B). DNA content analysis revealed small changes in the cell cycle distribution or apoptotic cell death upon pre-incubation with E8F08 (Figure S1A). Because of our interest in a decrease of recombination we proceeded with fraction E8F08 and subjected it to reverse-phase chromatography by reverse-phase chromatography. Second round screening of the resulting 44 fractions identified fraction 11 with the most pronounced decrease in recombination (Figure S1B). MALDI mass spectrometry analysis and Edman Sequencing identified a highly purified peptide in the protective fraction 11 with a molecular mass of 2,862 daltons and a theoretical isoelectric point (pl) of 6.23, encompassing the 27 amino acids MADEAGSEADHGETHSKRGHAKSRPVE27 (Fo27) (Figure 1C).

RESULTS

Identification of Hemofiltrate-Derived Peptide Fo27 That Mitigates Chemotherapy-Induced Rearrangements Underlying Development of Secondary Leukemia

To identify a human peptide which protects against leukemic MLLbcr rearrangements during chemotherapeutic treatment, we screened a hemofiltrate-derived library from patients suffering from renal failure (33, 37). During hemofiltration, patients’ blood was filtered through a membrane with a 20-30 kDa cut-off and the remaining peptides and small proteins were separated by cation exchange and reverse-phase high-performance liquid chromatography (HPLC) as outlined in Figure 1A (33). For functional analysis of the resulting 384 fractions (eight pH pool eluates each containing 48 fractions), cells carrying a chromosomally integrated EGFP-based reporter construct for MLLbcr rearrangements (30) were pre-treated with each eluate for 4 h and subsequently exposed to the chemotherapeutic drug etoposide (10 µM) for 72 h. Etoposide treatment conditions were previously established and demonstrated to induce a one order of magnitude increase of MLLbcr rearrangements in K562MLL and WTK1MLL reporter cells derived from human erythroleukemia and human lymphoblastoid cells, respectively (30). The 45 fractions showing significant differences in recombination frequency by χ² test were used to obtain additional measurements enabling more strict statistical analysis. This identified three fractions inducing statistically significant changes, namely E5F40 as well as E6F16 with stimulatory and, most interestingly, E8F08 with inhibitory effects protecting against MLLbcr rearrangements (Figure 1B). DNA content analysis revealed small changes in the cell cycle distribution or apoptotic cell death upon pre-incubation with E8F08 (Figure S1A). Because of our interest in a decrease of recombination we proceeded with fraction E8F08 and subjected it to reverse-phase chromatography by reverse-phase chromatography. Second round screening of the resulting 44 fractions identified fraction 11 with the most pronounced decrease in recombination (Figure S1B). MALDI mass spectrometry analysis and Edman Sequencing identified a highly purified peptide in the protective fraction 11 with a molecular mass of 2,862 daltons and a theoretical isoelectric point (pl) of 6.23, encompassing the 27 amino acids MADEAGSEADHGETHSKRGHAKSRPVE27 (Fo27) (Figure 1C).

Fo27 Differentially Affects MLLbcr Rearrangements After Treatment With Etoposide and Doxorubicin

To examine the efficacy of chemically synthesized Fo27, we pre-incubated the reporter line K562MLL for 4 h with increasing peptide concentrations before exposure to chemotherapeutic drugs according to the treatment protocols established in our earlier investigation (30). Serum-free AIM V™ Medium,
alleviating potential peptide proteolysis in FCS, improved the efficacy of the peptide, so that lower Fα27 concentrations were required for desired results and serum-free culture conditions were chosen for further experiments (data not shown). In this way we found that Fα27 reduced recombination frequencies in the chromosomally integrated MLLbcr reporter in K562MLL cells after etoposide (10 µM) or doxorubicin (2 µM) treatment with IC50 values for Fα27 of 15.2 µg/ml (5.3 µM) and 7.2 µg/ml (2.5 µM), respectively.

To exclude a mere cell line-dependent effect of Fα27 on chemotherapy-induced MLLbcr rearrangements, we examined another reporter line, namely WTK1MLL. Therefore, we pre-
incubated the cells with Fα27 followed by combined treatment with Fα27 plus etoposide or doxorubicin. Fα27 significantly decreased etoposide-induced recombination at the MLLbcr by 38%, but did not alter recombination in the presence of doxorubicin (Figure 2A). While etoposide forms covalent topoisomerase poisoning complexes, doxorubicin intercalates into the double helix (38, 39). We therefore wondered whether doxorubicin interferes with the enzymatic machinery processing DNA lesions or participating in EGFP reconstitution within the MLLbcr reporter. Consequently, we modified our treatment protocol such that WTK1MLL cells were washed and released into fresh medium after sequential pre-incubations with Fα27 and Fα27 plus doxorubicin (Figure 2B). Under these conditions FACS measurements indicated a significant decrease of recombination by 50%.

To exclude potential confounding factors, we assessed survival by quantification of live cells in the FSC/SSC gate during FACS measurements of EGFP-positive cells in these live cell populations. Figures S2A, B reveal that cell death induced by etoposide or doxorubicin treatments was not influenced by Fα27 applied at concentrations of 1 mg/ml and 10 µg/ml, respectively. Additionally, we tested the specificity of the effect of Fα27 by comparison with two scrambled peptides (Figure S2C). Peptide Scramble 1 was randomly composed of amino acids reflecting the average frequency of occurrence, whereas peptide Scramble 2 consisted of the same amino acids as Fα27 in a different order. When compared to these two peptides Fα27 again caused reduced recombination after doxorubicin treatment, namely by 53% and 37%, providing evidence for a specific effect.

Altogether, we verified that chemically synthesized Fα27, but not its scrambled counterpart protected against chemotherapy-induced MLLbcr rearrangements in different cell types without affecting cell death. Interestingly, the MLLbcr protective effect differed between a treatment with the cytostatic drug etoposide or doxorubicin, suggesting an impact of the respective mode-of-action.

Fα27 Has a Moderate Effect on DSB Repair That Can Partially Explain Protection From MLLbcr Rearrangements

DSBs are the predominating genotoxic lesions caused by etoposide, whereas doxorubicin induces a larger spectrum of DNA lesions (38, 39). To interrogate the possibility that Fα27 affects DSB repair, we engaged EGFP-based reporter constructs for the analysis of specific DSB repair pathways following
targeted cleavage by the endonuclease I-SceI. First, we measured microhomology-mediated end joining (MMEJ), and in a second set of experiments, homologous repair covering the pathways single-strand-annealing (SSA) and homologous recombination (HR) in WTK1 cells. For the analysis we pre-incubated the cells for 4 h with Fox27 and then introduced the repair constructs for MMEJ and homologous repair together with I-SceI expression construct by electroporation. This was followed by cultivation in Fox27-containing medium in analogy to the experimental conditions established for treatment with etoposide, the drug mostly inducing DSBs. Both MMEJ and homologous repair did not show statistically significant differences after Fox27 treatment (Figures 3A, B). To examine DSB repair in the chromosomal context and in the same reporter construct that served to measure MLLbcr rearrangements, we expressed I-SceI in WTK1MLL cells. The chromosomally integrated reporter comprises an I-SceI recognition site next to the MLLbcr. Under these conditions Fox27 induced a 20% reduction of I-SceI induced repair (Figure 3C). As seen before for chemotherapeutic drug exposures (Figure S2), the fraction of surviving cells was the same with and without Fox27 treatment (Figure 3, right panels) when targeted DSBs were introduced.

These results revealed that Fox27 downregulates the repair mechanism of DSBs to a lesser extent than MLLbcr.

**FIGURE 3** | Influence of Fox27 on I-SceI-induced MMEJ and homologous repair. (A) Scheme and results of MMEJ analysis following repair substrate cleavage by I-SceI. WTK1 cells were pre-treated with 1 mg/ml Fox27 for 4 h and then transfected with two plasmids, namely the repair construct for MMEJ and the I-SceI endonuclease expression plasmid (18). Targeted cleavage by I-SceI splits the mutated EJ-EGFP gene, so that MMEJ repair of the reporter permits EGFP reconstitution via pairing of adjacent microhomologies. EGFP-positive cells were measured by FACS 48 h post-transfection (n=9 obtained in three individual experiments). Mean recombination frequencies of controls were set to 100% each (absolute mean: 2.9x10⁻⁵). (B) Scheme and results of homologous repair analysis following cleavage by I-SceI. After cleavage by I-SceI either HR or SSA can reconstitute an intact EGFP gene. A 4 h pre-treatment with 1 mg/ml Fox27 and transfection with homologous repair reporter as well as I-SceI expression plasmid was followed by 48 h cultivation and FACS analysis (n=8-9 from three individual experiments). Mean recombination frequencies of controls were set to 100% each (absolute mean: 7.1x10⁻⁴). (C) Repair of I-SceI-induced DSBs in the same chromosomally integrated construct also used for detection of drug-induced MLLbcr rearrangements. WTK1MLL cells were treated with 1 mg/ml Fox27 for 4h, transfected with I-SceI expression plasmid and FACS analysis performed 48 h post-transfection (n=8-9 obtained in three independent experiments). Mean recombination frequencies of controls were set to 100% each (absolute mean: 1.2x10⁻⁴). All data represent mean ±SEM (*p < 0.05). Corresponding survival data obtained by FACS analysis of live cells in the FSC/SSC plot are displayed in the right panel each.
rearrangements (Figure 2) suggesting that another component contributing to Fo27’s protective effect still had to be discovered.

**Focal Accumulation of EndoG in the Nucleus and Local Binding to the MLLbcr Is Decreased by Fo27**

Previous works provided evidence for an increase of the mitochondrial nuclease EndoG in the nucleus and a critical involvement of this enzyme in the specific cleavage of the MLLbcr during replication stress such as following treatment with the cytostatic drug etoposide (20, 40, 41). Therefore, we examined the impact of Fo27 on these EndoG functions. To firstly verify the accumulation of DNA lesions in the nucleus due to the chemotherapeutic treatments, we immunostained WTK1 cells using antibodies directed against the damage marker γH2AX. Both exposures to etoposide and doxorubicin for 4 h led to sharp rises of nuclear γH2AX signals which were not influenced by Fo27 (Figure 4A). When we performed immunofluorescence microscopy with anti-EndoG antibodies, we detected distinct EndoG foci in the nucleus accumulating after the chemotherapeutic treatments (Figure 4B). Strikingly, pre-incubation with Fo27 peptide decreased nuclear EndoG signals down to the basal level in untreated cells. To exclude a cell cycle dependent effect of EndoG localization, we performed cell cycle analysis under the conditions of the immunofluorescence microscopic analyses. Figure S3 reveals that Fo27 pre-incubation did not alter the cell cycle distribution or percentage of apoptotic cells in cells treated with etoposide or doxorubicin.

To analyze the local binding of EndoG on MLLbcr sequences we performed chromatin immunoprecipitations (ChIPs) with anti-EndoG antibodies in WTK1MLL cells. PCR amplification of precipitated genomic DNA was specific for the MLLbcr region inserted in the chromosomally integrated reporter sequence (Figure 4C). WTK1MLL cells showed an increase of EndoG binding to the MLLbcr sequence after treatment with doxorubicin (Figure 4D), which was decreased to basal levels by Fo27 peptide pre-treatment.

All-in-all, the peptide Fo27 affects the localization of EndoG in the nucleus and at the MLLbcr sequence without any influence on the global DNA damage.

**Localization of the Fo27 Peptide**

To discriminate between a potential extracellular vs. intracellular mode-of-action of Fo27, we visualized its cellular localization upon addition to the culture medium. For these experiments we used adherent HeLa cells optimal for microscopy. We previously demonstrated that HeLa cells generate comparable results to cells from the hematopoietic system in siRNA screenings, functional and biochemical analyses and thus serve as a representative cell model for the processes leading to MLLbcr rearrangements (19, 20). We used chemically synthesized Fo27 N-terminally labeled with a TAMRA dye, which is membrane permeable and does not interfere with cellular entry of the peptide. First, spinning disc confocal microscopy revealed dark cells compared to bright, TAMRA dye-positive medium, strongly indicating that Fo27 is unable to cross the cell membrane (Figures 5A, B). Second, HILO microscopy, which enables single molecule sensitivity by reducing autofluorescent background of the cell via sheet-like illumination (36), allowed us to exclude the presence of potentially active trace amounts of Fo27 in the cellular interior (Figures 5C, D). Occasionally, we observed individual labeled peptides associating transiently with the cell membrane.

Overall, these experiments suggested that Fo27 acts by extracellular signaling.

**Fo27 Acts via NFκB and Regulates TLR4 Target Genes**

Landers et al. (42) reported that fibrinogen, particularly the fibrinogen α chain, is selectively cleaved by proteinases and resulting cleavage products can bind TLR4 and regulate downstream transcription factors like nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB). Though fibrinogen α fragments identified by Landers and colleagues were not identical with Fo27, we were inspired by these findings and therefore analyzed involvement of NFκB signaling in the pathway regulated by Fo27 by use of the NFκB inhibitor disulfiram (43). Disulfiram was added to the medium of WTK1MLL cell cultures as the first pre-treatment, i.e. 5 h before sequential Fo27 and Fo27 plus doxorubicin pre-incubations followed by release into fresh medium (Figure 6A). Recombination measurements showed similar basal frequencies and increases in doxorubicin-exposed WTK1MLL cells with and without disulfiram (Figure 6B). Fo27 exposure reduced the recombination frequency in the absence but no longer in the presence of disulfiram (Figure 6B). The Fo27 effect on recombination was also abrogated in etoposide-exposed cells, even though combined disulfiram and etoposide treatment stimulated etoposide-induced MLLbcr rearrangements and reduced survival even further compared with single etoposide treatment (Figure S4).

Of note, accumulation of DNA damage and/or compensatory use of alternative DSB repair pathways may explain why MLLbcr rearrangements continued after inhibition of NFκB, which promotes non-homologous end joining (NHEJ) and HR (18, 31).

Given that ROS trigger NFκB signaling (44, 45) and that ROS production is one of the major toxic effects of chemotherapeutic treatments, doxorubicin in particular (46), we investigated a potential role of ROS in the Fo27 effect. When exposing the cells to N-acetylcysteine (NAC), a superoxide scavenger (47) that decreases ROS production, the Fo27-dependent decrease in the recombination frequency of doxorubicin-treated WTK1MLL cells was no longer detectable (Figure S5).

To test the hypothesis that proinflammatory signaling by TLR4 and NFκB is influenced by Fo27, we checked the expression of characteristic cytokines downstream of this pathway. The mRNA levels of interleukin (IL)-6, IL-8 and interferon (IFN)γ all increased after doxorubicin treatment and decreased following Fo27 pre-treatment (Figure 6C). The TLR4 mRNA expression level was not changed under these conditions.

Altogether, both recombination measurements when inhibiting NFκB and analysis of TLR4 activated genes support our hypothesis that TLR4 signaling is blocked by Fo27.
FIGURE 4 | Fa27 influences EndoG accumulation in nuclear foci during treatment and reduces local EndoG binding to the MLLbcr. (A) No effect of Fa27 on nuclear DNA damage level during chemotherapeutic treatment. WTK1 cells were treated for 4 h with 1 mg/ml Fa27 followed by inclusion of 10 µM etoposide for 4 h or 10 µg/ml Fa27 for 4 h followed by inclusion of 0.5 µM doxorubicin for 4 h. Fixed cells showed an increase of cells with ≥12 γH2AX foci/nucleus after etoposide or doxorubicin treatment but no decrease with Fa27 pre-treatment (n=4 obtained in two independent experiments). (B) Decrease of nuclear EndoG signals by Fa27 treatment. WTK1 cells were treated as described in (A) and immunofluorescently labeled for EndoG detection. The nuclear EndoG signals increased after etoposide (n=294-463 obtained in two independent experiments) or doxorubicin (n=296-485 obtained in two independent experiments) treatments and Fa27 significantly decreased the nuclear EndoG level. Insets display the highlighted region at two-fold magnification. Scale bars indicate 10 µM. White dashed lines encircle the DAPI-stained nucleus. All values represent mean ±SEM (*p < 0.05; ****p < 0.0001). (C) Scheme of the MLLbcr region flanked by two mutated EGFP genes. The scheme visualizes the positions of the primers used for PCR after chromatin immunoprecipitation (ChIP). To analyze the binding of EndoG to the MLLbcr a ChIP was performed. Sonified DNA bound by proteins was immunoprecipitated with EndoG antibody or incubated with a control antibody. After DNA isolation a PCR was performed and band intensities evaluated. (D) Decreased EndoG binding to the MLLbcr after Fa27 treatment. WTK1/MLL cells were treated for 4 h with 10 µg/ml Fa27 and additionally with 0.5 µM doxorubicin for 4 h. Two independent experiments for EndoG-ChIP and IgG-ChIP were performed. After PCR amplification of the MLLbcr in the recombination reporter, band intensities for input DNA and EndoG-ChIP samples after doxorubicin treatment were set to 100% and relative intensities calculated for the other samples including IgG-ChIP detected on the same membrane and with the same exposure time. EndoG binding to MLLbcr was increased after doxorubicin treatment and decreased with Fa27 treatment. Neither the input nor the control showed such a pattern. Control GAPDH PCR was positive for input but negative for ChIP samples. Data are shown as mean ±SD. See also Figure S3.
F27 Antagonizes LPS-Mediated TLR4 Activation

To examine whether F27 directly targets TLR4, we studied the impact of F27 on TLR4 signaling after extracellular stimulation by the well-established TLR4 agonist lipopolysaccharide (LPS). To this end we engaged HEK-Blue™ hTLR4 reporter cells enabling TLR4 activity measurements via a color change due to inducible release of SEAP (secreted embryonic alkaline phosphatase). Our results showed that pre-treatment with F27 decreased LPS stimulation significantly at different peptide concentrations by 19% to 32% (Figure 7A). For comparison, when HEK-Blue™ hTLR4 cells were pre-treated with Scramble 2, composed of the F27 amino acids in a random order, LPS stimulation was not significantly compromised (Figure S6A).

To examine a potential influence of F27 on TLR4 signaling during chemotherapeutic treatment, we treated HEK-Blue™ hTLR4 cells with doxorubicin after pre-treatment with active or control peptides (Figure S6B). Doxorubicin treatment on its own did not affect the release of the reporter SEAP. In the presence of peptides, we observed a statistically significant decrease of TLR4 signaling by 21% when comparing F27 versus Scramble 2 pre-treated cells.

In summary, F27 antagonizes TLR4 activation by LPS in a sequence-specific fashion, suggesting a direct interaction of F27 with TLR4.

Detection of a Close Proximity Between TLR4 and F27 In Situ

To test the hypothesis of an association of F27 with TLR4, we examined whether they can be found in close proximity to each other. Therefore, we labeled F27 either N- or C-terminally with a Flag-Tag, which was separated from the peptide by a GSSGSS-linker each. Proximity ligation assay (PLA) studies with HEK-Blue™ hTLR4 cells revealed sites of proximity between TLR4 and both F27 versions spread over the cellular area, similarly as seen for TLR4 and its co-receptor myeloid differentiation 2 (MD-2).
To strengthen this result, the corresponding experiment was also performed with WTK1 cells showing comparable results (Figure S7). For comparison, PLA signals for the DNA repair proteins Ku70 and Ku80 were limited to the DAPI-stained nucleus. Raising the concentration of tagged Fα27 increased the average numbers of foci per cell, reaching statistical significance after incubation with C-terminally tagged peptide Fα27-Flag.

Human Hematopoietic Stem and Progenitor Cells Show Increased Breakage of the MLLbcR Under Doxorubicin Treatment Which Is Prevented by Fα27

Hematopoietic stem and progenitor cells harbor the cells-of-origin of leukemia (48, 49) and express TLRs and their co-receptors (50). To examine the impact of Fα27 on MLLbcR stability in these cells, we treated primary human cord blood-derived HSPCs with doxorubicin at the IC80 dose of 0.072 µM. Treatment of HSPCs with doxorubicin caused breakage of approximately half of the MLLbcR sites, i.e. caused by 40% reduced band intensities after genomic PCR amplification of the MLLbcR as compared to the distant intron 20 control site within the MLL gene (Figure 8A). Strikingly, pre-treatment with Fα27 reduced breakage as reflected by a 13% reduction of the PCR band intensities only. From this we concluded that Fα27 not only prevents MLLbcR rearrangements in immortalized cells derived from the human hematopoietic system but also protects MLLbcR from breakage in primary human HSPCs.

DISCUSSION

In this study, we screened a hemofiltrate-derived library for peptides decreasing leukemic MLLbcR rearrangements and identified a peptide with 27 amino acids derived from the C-terminus of the fibrinogen α chain (Fα27). Fibrinogen,
composed of the α, β and γ chains, is up-regulated and cleaved during inflammation as well as after vascular injury to form fibrin, a component of blood clots (55, 56). Further reports described increased expression of fibrinogen in a large variety of tumors (57–59) and correlation of high fibrinogen plasma levels with cancer risk, worse disease-free and poor overall survival (59–61). Peptides derived from the C-terminus of fibrinogen α (62–64), including a peptide identical to Fo27 (65), were also identified during clinical association studies in search of serum-derived tumor biomarkers. Notably, one study focusing on acute myeloid leukemia (AML) showed that a 38 amino acid long C-terminal fragment of fibrinogen α encompassing Fo27 was found to be reduced in sera from newly diagnosed, refractory and relapsed cases and associated with improved overall survival of patients, which was the opposite of what was observed for the fibrinogen α level (62). In our work, we discovered Fo27 to show sequence specific bioactivity with IC50 values in the lower micromolar, i.e. the physiological range of fibrinogen (66). Importantly, we provide evidence that Fo27 protects against MLLbcr breakage and rearrangements triggering AML, but not against global DNA damage or cell death induced by cytostatic treatments and therefore holds promise to ameliorate side effects without compromising the therapeutic achievement.

After identification of the bioactive peptide in the circulating blood peptidome we confirmed functional equivalence of its synthetic counterpart and analyzed its mode-of-action.

FIGURE 7 | Fo27 binds TLR4 and reduces LPS stimulation. (A) Fo27 inhibits TLR4 activation by LPS. Scheme of experimental analysis of TLR4 signaling and results obtained in HEK-Blue hTLR4 cells pre-treated for 2 h with different concentrations of Fo27 followed by additional treatment with 10 ng/ml LPS for 10 h. TLR4 stimulation led to a release of secreted embryonic alkaline phosphatase (SEAP) detected at OD 655 nm. LPS treatment increased SEAP release which was reduced by Fo27 pre-treatment. Mean OD values of LPS-treated controls without peptide were set to 100% in each experiment. Data (n=12 obtained in two experiments) are shown as mean +SEM and statistics were calculated relative to LPS stimulation without peptide (*p < 0.05; **p < 0.01; ****p < 0.0001). (B) TLR4 and Fo27 interaction. HEK-Blue hTLR4 cells were seeded 72 h before the experiment and treated for 2 h with N-terminally tagged Flag-Fo27, C-terminally tagged Fo27-Flag or water (ctrl). PLA analysis showed an interaction between both tagged Fo27 peptides and the TLR4. Two positive controls are shown: PLA for TLR4 and its binding partner MD-2 on the cellular surface and for Ku70-Ku80 complexes in the nucleus. The negative control was done with PLA substrates but without antibodies. Margins of cells are marked by white stippled lines and the scale bar indicates 10 µM (n=187-425 from two independent experiments each; mean +SEM; ****p < 0.0001). See also Figure S6.
Wide field image microscopy of TAMRA-labeled Fo27 showed that the peptide was not entering the cell. Therefore, we considered that Fo27 acts via a membrane receptor. Smiley and colleagues (67) reported that fibrinogen activates TLR4 on macrophages. More recent work by Landers and colleagues (42) showed that fibrinogen α undergoes proteolytic processing releasing C-terminal regions during innate immune responses and generating cleavage products that can directly bind TLR4. In this way fibrinogen, a drug with a broad spectrum of cell damage, intercalates into the DNA, which promotes translocation of EndoG from mitochondria to the nucleus. Nuclear EndoG cleaves the MLL gene predicted to form pronounced secondary structures. Stimulation of TLR4, such as by LPS, was as well reported to increase ROS production in mitochondria (54), again inducing DNA damage. Nuclear EndoG accumulation in the nucleus is reduced and the MLL gene is protected from breakage.

A variety of molecules activate TLRs on the cell surface (69). TLR4 is best known to be targeted by LPS from bacteria, which leads to cytokine production (70). We therefore analyzed the effect of Fo27 on LPS-activated TLR4 signaling using a reporter cell line and indeed noticed an inhibitory effect of the peptide in the concentration range exerting MLLbrc protection. Importantly, scrambled peptide was ineffective excluding that simple physicochemical characteristics of Fo27 underly the inhibitory interaction such as known from positively charged antimicrobial peptides (71).

FIGURE 8 | Fo27 prevents MLLbrc breakage in HSPCs. (A) MLLbrc breakage analysis after doxorubicin and Fo27 treatment of HSPCs. HSPCs were treated 4 h with 10 µg/ml Fo27 followed by 4 h of treatment with Fo27 plus 0.072 µM doxorubicin. After genomic DNA isolation breakage was assessed by PCR targeting the endogenous MLLbrc locus. Doxorubicin treatment increased breakage as indicated by a 40% decrease of the band intensity, which was reduced in Fo27 pre-treated cells by only 13%. Data from five independent experiments are shown as mean ± SEM (Friedman-Test indicating p=0.0087, followed by Wilcoxon test). The framed image shows MLLbrc PCR amplifications from the same gel exposed with the same intensity and cropped at the dotted line. MLL intron20 was amplified as control. (B) Model of MLLbrc protection by Fo27. LPS activation of TLR4 leads to NFκB-mediated gene expression of DNA repair genes and proinflammatory cytokines like type I interferons (51). The interferon IFNγ can increase EndoG release from the mitochondria (62). Etoposide, a topoisomerase II inhibitor, induces DNA damage and EndoG release from the mitochondria (53). Doxorubicin, a drug with a broad spectrum of cell damage, intercalates into the DNA, which promotes translocation of EndoG from mitochondria to the nucleus. Nuclear EndoG cleaves the MLLbrc predicted to form pronounced secondary structures. Stimulation of TLR4, such as by LPS, was as well reported to increase ROS production in mitochondria (54), again inducing DNA damage. We show that Fo27 blocks TLR4 activation and downstream NFκB signaling. Furthermore, EndoG accumulation in the nucleus is reduced and the MLL gene is protected from breakage.

Findings made in this work are indicated in red color.

First, dampening the NFκB pathway compromises DSB repair via NHEJ and HR, two pathways contributing to
chromosomal rearrangements (18, 19, 31). In our study, use of the pharmacological inhibitor disulfiram showed that Fo27 targets the NFkB pathway when counteracting etoposide- and doxorubicin-induced MLLbcr rearrangements. Analysis of I-Scel-induced repair revealed that Fo27 affects the DSB repair process itself, yet moderately, and therefore can only partially explain Fo27 mode-of-action. Yet, among the different DSB repair genes NFkB transcriptionally up-regulates also ATM, which not only regulates DSB repair but also promotes RNF20-RNF40-mediated H2B ubiquitination and chromatin remodeling as a prerequisite for EndoG loading onto the MLLbcr (20). Thus, the MLLbcr protective effect of Fo27 may as well influences NFkB’s involvement in other cellular activities ranging from chromatin remodeling to cytokine production.

Second, inflammatory signaling is known to generate ROS (72, 73), inducing transport of EndoG from mitochondria to the nucleus, where EndoG binds and cleaves the MLLbcr (19). Along this line, the proinflammatory cytokine IFNγ was reported to promote EndoG release from mitochondria (52) following TLR4 activation. Here, we observed that Fo27 inhibits LPS-activated TLR4 signaling and expression of the proinflammatory cytokines IL-6, IL-8 and IFNγ. Therefore, blocking inflammatory signaling and ROS production represents another mechanism contributing to MLLbcr protection by Fo27.

Moreover, chemotherapeutic treatment itself causes the accumulation of ROS. Particularly doxorubicin boosts ROS formation in mitochondria (21, 39), but also etoposide was shown to shuttle EndoG into the nucleus (53). In response to chemotherapeutic treatment, we saw focal accumulation of EndoG in the nucleus and MLLbcr binding, which was counteracted by Fo27. In the presence of the ROS scavenger NAC, treatment-induced rearrangements at the MLLbcr were no longer suppressed by Fo27. The peptide may therefore break a loop amplifying ROS production by the chemotherapeutic treatment and TLR4 signaling.

What is the relative importance of these mechanisms underlying the protective mode-of-action of Fo27? Clues may come from the activities of cells treated with etoposide versus doxorubicin. While both drugs are topoisomerase II inhibitors, etoposide forms topoisomerase poisoning complexes, blocking DNA resealing during the enzymatic reaction, whereas doxorubicin intercalates into the double helix compromising topoisomerase functions, RNA- and DNA synthesis as well as generating free oxygen radicals (38, 39). Therefore, etoposide primarily induces DSBs, while doxorubicin additionally causes formation of oxidative DNA lesions, DNA adducts and RNA-DNA hybrids, representing obstacles for nascent DNA synthesis (74). Interestingly, when we studied MLLbcr rearrangements following a short (4 h) treatment period and a long (72 h) recovery period versus uninterrupted treatment, a recovery period was necessary to unveil the protective effect of Fo27 during doxorubicin but not etoposide treatment. These results suggested that Fo27 antagonizes formation of doxorubicin-induced DNA damage at the MLLbcr rather than its repair, while in case of etoposide the impact of Fo27 on the repair of drug-induced DSBs at the MLLbcr is as well significant. Yet, the effect of the peptide on DSB repair following targeted cleavage of the chromosomal reporter constructs was moderate, which may explain why reduction of MLLbcr rearrangements required a higher peptide concentration after etoposide than after doxorubicin exposures. Accordingly, we propose that Fo27 is most potent in MLLbcr protection, when the MLLbcr is challenged by synergistically destabilizing mechanisms such as during doxorubicin treatment.

Knowing that Fo27’s effect on the MLLbcr involves oxidative stress and proinflammatory signaling via TLR4 and NFkB, how then are the two pathways connected? In this context reports showing up-regulation of the expression level of TLR4 by doxorubicin were of interest (75, 76). This upregulated level of TLR4, though not reaching statistical significance at early time points in mice or when comparing patient cohorts, has been discussed to contribute to toxic side effects in chemotherapy like cardiomyopathy (76). Moreover, published work demonstrated that doxorubicin-mediated DNA damage results in the production of single-stranded DNA that directly activates the DNA-sensing cGAS-STING pathway of IFN induction (77). Indeed, TLRs are not only activated by so-called pathogen-associated molecular patterns (PAMPs) but also by damage-associated molecular patterns (DAMPs). While PAMPs like bacterial LPS and virus-derived nucleic acids stem from exogenous sources, namely microbiological infections, DAMPs are intracellular molecules such as host DNA or fibrinogen, which are released by activated or dying cells (72, 78). Though conflicting evidence regarding the precise binding sites of PAMPs and DAMPs exist, there is a possibility that LPS and fibrinogen compete for binding to the same binding site on TLR4. Doxorubicin treatment therefore triggers TLR4 signaling via DAMPs from dying cells as well as via upregulation of the receptor itself. Inflammatory TLR4 signaling will further increase tissue damage and therefore DAMPs. In this way, an amplification loop of signals destabilizing the MLLbcr through IFN and extra ROS generated during TLR4 signaling is created, which ultimately shuttles EndoG to the nucleus where it specifically cleaves hard-to-replicate sites such as the MLLbcr in surviving cells (20). Importantly, HSPCs are highly sensitive to products which activate TLRs and respond to type I and II interferons, like IFNγ. Consistently, we observed that Fo27 pre-treatment ameliorates MLLbcr breakage in HSPCs during doxorubicin chemotherapy. After bone marrow transplantation 13% of the patients were reported to develop a secondary malignancy in the first 15 years after treatment (79). Breaking the cascade of proinflammatory signaling events directly at the receptor via an antagonistic fibrinogen fragment therefore has potential to specifically reduce the side effect of leukemic rearrangements in the cells-of-origin of leukemia.

Our work provides proof-of-concept that genome destabilizing events causing secondary leukemia can be prevented by a peptide blocking TLR4 activation. Since Fo27 is an endogenous peptide, it is conceivable that an autoregulatory feedback loop involving Fo27 and related fibrinogen α-derived peptides exists. Thus, at sites of tissue injury and inflammation upregulation of DAMPs like fibrinogen will activate TLR4 to
initiate inflammation; proteolytic cleavage products of fibrinogen may thereafter antagonize TLR4 activation and terminate inflammation. Loss of such control mechanisms will deregulate TLR signaling and contribute to the persistence of inflammatory responses underlying chronic diseases (80, 81). Persistent DAMP accumulation and inflammation are associated with cancer (72). TLR4 signaling has been shown to enhance the proliferation and migration of malignant tumor cells in patients (82, 83), and overexpression in tumors was correlated with metastasis and chemoresistance (84). Our work has provided evidence for even another detrimental outcome of TLR4 signaling in cancer patients undergoing chemotherapy, i.e. leukemia-inducing chromosomal damage in healthy HSPCs without killing the cells. Given that TLR4 is unique among TLRs in terms of its MD-2 co-receptor requirement (85), it has increasingly gained interest as a target of therapeutic strategies engaging small molecules (72, 86, 87). Since Fz27 is an endogenous inhibitor of TLR4 signaling that may have undergone natural selection processes, Fz27 could be of interest as a lead compound that may offer a means to selectively inhibit the DAMP-induced TLR4 activation loop causing secondary leukemia in cancer patients undergoing chemotherapy.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by University of Ulm. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

**AUTHOR CONTRIBUTIONS**

LW conceived the study. RW and BG performed the screen. RW and LM established assay conditions and performed initial experiments. LW and JE designed the experiments. JE performed the experiments and processed the data with help of RW. JG and AP designed, performed and interpreted spinning disc confocal and Hilo microscopy experiments. LS, JM, and WF were in charge of the peptide library and peptide identification. LW and JE interpreted the data and designed the manuscript. JE generated the figures and wrote the initial draft of the manuscript, which was revised by LW. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.689063/full#supplementary-material

**REFERENCES**

1. De Braekeleer M, Morel F, Le Bris MJ, Herry A, Douet-Guilbert N. The MLL Recombinome of Acute Leukemias in 2017. *Leukemia* (2018) 32:273–84. doi: 10.1038/leu.2017.213
2. Göke B, Wiesmüller L. Leukemogenic Rearrangements at the Mixed Lineage Leukemia Gene (MLL)—Multiple Rather Than a Single Mechanism. *Front Cell Dev Biol* (2015) 3:41. doi: 10.3389/fcell.2015.00041
3. Meyer C, Burmeister T, Gröger D, Tsaur G, Fasehina L, Renneville A, et al. The MLL Recombinome of Acute Leukemias in 2017. *Leukemia* (2018) 32:273–84. doi: 10.1038/leu.2017.213
4. Liu H, Cheng EHY, Hsieh JJD. MLL Fusions: Pathways to Leukemia. *Cancer Biol Ther* (2009) 8:1204–11. doi: 10.4161/cbt.8.13.8924
5. Glissoner B, Goekbuget N, Riedler H, Arnold R, Schwartz S, Diedrich H, et al. CD10- Pre-B Acute Lymphoblastic Leukemia (ALL) Is a Distinct High-Risk Subgroup of Adult ALL Associated With a High Frequency of MLL Aberrations: Results of the German Multicenter Trials for Adult ALL (GMALL). *Blood* (2005) 106:4054–6. doi: 10.1182/blood-2005-05-1866
6. Emerenciano M, Meyer C, Mansur MB, Marschalek R, Pombo-De-Oliveira MS. The Distribution of MLL Breakpoints Correlates With Outcome in Infant Acute Leukaemia. *Br J Haematol* (2013) 161:224–36. doi: 10.1111/bjh.12250
7. Strick R, Strissel PL, Borgers S, Smith SL, Rowley JD. Dietary Flavonoids Induce Cleavage in the MLL Gene and May Contribute to Infant Leukemia. *Proc Natl Acad Sci USA* (2000) 97:4790–5. doi: 10.1073/pnas.070061297
8. Van Waalwijk Van Doorn-Khosrovani SB, Janssen J, Maas LM, Godschalk RWL, Nijhuis JG, Van Schooten FJ. Dietary Flavonoids Induce MLL
47. Aruoma OI, Halliwell B, Boey BM, Butler J. The Antioxidant Action of N-acetylcycteine: Its Reaction With Hydrogen Peroxide, Hydroxyl Radical, Superoxide, and Hypochlorous Acid. Free Radical Biol Med (1989) 7:693–9. doi: 10.1016/0891-5849(89)90066-X.
48. Durante M, Cucinotta FA. Heavy Ion Carcinogenesis and Human Space Exploration. Nat Rev Cancer (2008) 8:465–72. doi: 10.1038/nrc2391.
49. Barcellos-Hoff MH, Blakely EA, Burma S, Fornace AJ Jr., Gerson S, Hlatky L, Zhdanov DD, Gladilina YA, Pokrovsky VS, Grishin DV, Grachev VA, Orlova Kuzmich NN, Sivak VK, Chubarev NV, Porozov BY, Savateeva-Lyubimova.
50. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, et al. Toll-Like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment. Immunity (2006) 24:801–12. doi: 10.1016/j.immuni.2006.04.008.
51. Kuzmin NH, Sivak VK, Chubarev NV, Porozov BY, Savateeva-Lyubimova NT, Peri F. Th4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. Vaccines (2017) 5:34. doi: 10.3390/vaccines5040034.
52. Zhdanov DD, Gladilina YA, Pokrovsky VS, Grishin DV, Grachev VA, Orlova VS, et al. Endonuclease G Modulates the Alternative Splicing of Deoxyribonuclease 1 mRNA in Human CD4(+)+ T Lymphocytes and Prevents the Progression of Apoptosis. Biochimie (2018) 157:158–76. doi: 10.1016/j.biochi.2018.11.020.
53. Bao W, Zhou X, Bao W, Chen Y, Cheng L, Qiu G, et al. Endonuclease G Promotes Cell Death of Non-Invasive Human Breast Cancer Cells. Exp Cell Res (2006) 312:4139–49. doi: 10.1016/j.yexcr.2006.09.012.
54. Yuan X, Zhou Y, Wang W, Li J, Xie G, Zhao Y, et al. Activation of TLR4 SignalingPromotes Gastric Cancer Progression by Inducing Mitochondrial ROS Production. Cell Death Dis (2013) 4:e794. doi: 10.1038/cddis03.1334.
55. Crabtree GR. The Molecular Biology of Fibrinogen. In: G Stamatoyannopoulos, AW Nienhaus, P Leder, PW Majerus, editors. The Molecular Basis of Blood Diseases. Philadelphia: W.B. Saunders (1987).
56. Herrick S, Blanc-Brude O, Gray A, Laurent G. Fibrinogen. Int J Biochem Cell Biol (1999) 31:741–6. doi: 10.1016/S1357-2799(00)00321-1.
57. Tao YL, Li T, Gao J, Liu ZG, TuZW, Li G, et al. Identifying FGA Peptides as Nasopharyngeal Carcinoma-Associated Biomarkers by Magnetic Beads. J Cell Biochem (2012) 113:2268–78. doi: 10.1002/jcb.24097.
58. Son H-J, Park JW, Chang HJ, Kim DY, Kim BC, Kim SY, et al. Preoperative Serum Peptide Biomarkers Correlates With the Disease States of Multiple Myeloma. Clin Proteomics (2019) 16:17. doi: 10.1186/s10034-013-2009-4.
59. Zhang D, Zhou Z, Hao W, Chen Y, Cheng L, Qiu G, et al. Plasma Fibrinogen Levels are Correlated With Postoperative Distant Metastasis and Prognosis in Esophageal Squamous Cell Carcinoma. Oncotarget (2015) 6:38410–20. doi: 10.18632/oncotarget.4800.
60. Yu W, Wang Y, Shen B. An Elevated Preoperative Plasma Fibrinogen Level is Associated With Poor Overall Survival in Chinese Gastric Cancer Patients. Cancer Epidemiol (2016) 42:39–45. doi: 10.1016/j.canep.2016.03.004.
61. Bai J, Yang Y, Wang W, Li J, Wang F, He A. Variability of Serum Novel Oligopeptide Biomarkers Correlates With the Disease States of Multiple Myeloma. J Cell Biochem (2012) 113:2268–78. doi: 10.1002/jcb.24097.
62. Bai J, Yang Y, Wang W, Li J, Wang F, He A. Variability of Serum Novel Oligopeptide Biomarkers Correlates With the Disease States of Multiple Myeloma. Clin Proteomics (2019) 16:17. doi: 10.1186/s10034-013-2009-4.
63. Bai J, Yang Y, Wang W, Li J, Wang F, He A. Variability of Serum Novel Oligopeptide Biomarkers Correlates With the Disease States of Multiple Myeloma. Clin Proteomics (2019) 16:17. doi: 10.1186/s10034-013-2009-4.
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