SUPPLEMENTARY APPENDIX

Targeting metabolism and survival in chronic lymphocytic leukemia and Richter syndrome cells by a novel NF-κB inhibitor

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Supplementary Methods

Nurse-like cells (NLC) generation

NLC were obtained from freshly purified or frozen CLL cells plated at high density ($10x10^6$/ml/well) in RPMI+10%FCS for 14 days.[1] Cell morphology and numbers after vehicle or IT-901 treatment were determined by Giemsa staining.

Antibodies used for immunofluorescence

Primary and secondary antibodies used for immunofluorescence were: -NF-kB p65 (clone F-6/NF-kB/p65, #sc-8008, Santa Cruz Biotechnology, Segrate, Italy), AlexaFluor 488-conjugated goat anti-mouse IgG (1:50) (ThermoFisher Scientific). Nuclei were counterstained with DAPI (ThermoFisher Scientific).

Antibodies used for flow cytometry

Antibodies used for flow cytometry were: anti-CD19 FITC (LT19), -CD19 PE (LT19), -CD19 APC (LT19) all from Miltenyi Biotech (Bologna, Italy), -CD5 FITC (UCHT2), -CD19 PeCy7 (SJ25CI) both from BD Pharmigen (Milan, Italy), -CD45 PerCP Cy5.5 (2D1), -CD49d PE (9F10), -CD274 PE (M1H1), -CD21 FITC (HB5) , -CD3 APC (OKT3) all from eBioscience, -ICAM1 AlexaFluor647 (HCD54), -VCAM1 PE (STA) all from BioLegend (Campoverde, Milan, Italy), -CD86 FITC (BU63, Caltag MedSystems, Buckingham, UK), -CD23 PE (Tu1, SouthernBiotech, Valter Occhiena, Turin, Italy), -CD38 AlexaFluor488 (AT1135, Serotec, Milan, Italy). Data were acquired using a FACSCanto II cytofluorimeter and processed with FACSDiva v8 (BD Biosciences, Milan, Italy) and FlowJo v10 softwares (TreeStar, Ashland OR).

Apoptosis

CLL, RS, normal B and T lymphocytes, CLL cell lines and HS-5 cells, were exposed to vehicle (DMSO) or increasing doses of IT-901 for different time points. At the end of the treatment, cells were collected and stained with AnnexinV FITC-labelled and Propidium Iodide (eBioscience) following manufacturer instructions and samples analyzed by flow cytometry with a BD FACSCanto II. Alternatively, an AnnexinV APC-labelled kit (eBioscience) was used.

Where indicated, N-acetyl cysteine was used as mROS scavenger: cells were pre-treated with NAC (5 mM, 30 minutes, 37 °C) before adding vehicle or IT-901.

Lysates, nuclear extracts and western blotting

CLL primary cells, where indicated, were cultured over a layer of stromal cells (HS-5) for 6 hours to maximize the NF-kB1/p65 pathway, in the presence of increasing doses of IT-901 or vehicle (a pre-culture of 30 minutes of the leukemic cells over the stromal layer before adding IT-901 was adopted to let CLL cells to interact with HS-5). After the culture, leukemic cells ($20x10^6$) were collected and cytosolic and nuclear fractions obtained using a nuclear extraction kit, following manufacturer' instructions (Nuclear extraction kit, Active motif, Firenze, Italy). CLL cell lines were not cultured on a stromal layer before purifying nuclear fraction because of the constitutive activation of the NF-kB1 pathway.

Total lysates were obtained by lysing CLL cells or cell lines as previously described.[2] Nuclear and cytosolic fractions, as well as total lysates were resolved by Bolt SDS-PAGE gels and transferred to nitrocellulose membranes (ThermoFisher Scientific). The following antibodies were used: anti-NF-kB1/p105-p50 (#3035), -p65 (#8242), -P-IkBα (#9246), -IkBα (#4814), -β-tubulin (#5346), -Lamin A/C (#4777), -Caspase 3 (#9662), -XIAP (#14334), -Bim (#2933) (all from Cell Signaling Technologies, Milan, Italy). Bands were detected with an HRP-conjugated goat anti-mouse IgG antibody (PerkinElmer) or anti-rabbit antibody (Santa Cruz Biotechnology). An anti-actin HRP-conjugated antibody was used as a loading control (sc-1616, Santa Cruz Biotechnology). β-tubulin and lamin A/C were used as cytosolic and nuclear markers, respectively. Blots were developed using enhanced chemiluminescence (GE Healthcare). Images were acquired with the ImageQuant LAS 4000 ChemiDoc (GE Healthcare) or membrane exposed to films (Amersham, GE Healthcare). Quantification of protein bands in western blot analysis were performed using the ImageJ software (downloadable at https://imagej.nih.gov/ij/)

NF-kB binding activity (ELISA)

NF-kB transcriptional activity was specifically measured using an ELISA-based microwell assay, which is at least 10 times more sensitive than electrophoretic mobility shift assay.[3] Nuclear proteins (7.5 μg) obtained from primary CLL and cell lines treated or not with IT-901, were assayed for p50, p65 and c-Rel DNA binding with a TransAM NF-kB ELISA Kit (Active Motif, Carlsbad, CA) according to the manufacturer' instructions. The consensus oligonucleotide, immobilized in each well, used for NF-kB binding was 5' -GGGACTTTCC-3'.

Mitochondrial reactive oxygen species (mROS) and mitochondrial inner membrane potential.

For mROS analyses, CLL cells were treated with increasing doses of IT-901 for 6 hours and then stained with the MitoSox Red kit (Life Technologies), following manufacturer' instructions and analyzed by flow cytometry.

Mitochondrial electrochemical membrane potential (ϕm) was assessed using the potentiometric probe JC-1 (1 μM, Life Technologies) by flow cytometry. CLL cells were exposed to vehicle or IT-901 (5-10 μM) for 6 hours before staining. Loss of the membrane potential ($\Delta\varphi$m) was calculated as percentage of cells showing low levels of JC-1 PE staining. Carbonyl cyanide m-chlorophenylhydrazone (CCCP, 50 μM*),* a mitochondrial uncoupler, was used as positive control. For CLL cell lines, IT-901 was used at 2.5 and 5 μM. Membrane potential on CLL cell lines was also evaluated using the tetramethylrodamine-methyl ester dye (TMRM, 20 nM, Molecular Probes, ThermoFisher Scientific). Briefly, cells were exposed to vehicle or IT-901 for 6 hours, stained and analyzed by flow cytometry. FCCP (1 μM) was used as positive control.

Confocal microscopy analyses

To study the activation of the NF-kB1/p65 pathway following the interaction between leukemic cells and the stromal component, NLC, cultured on coverslips, were starved for 48 hours in RPMI+2.5%FCS before being co-cultured with autologous CLL cells for 24 hours in the presence or absence of IT-901 (10 μM). NLC without CLL were used as negative control (Basal). After activation, CLL cells were washed away with PBS. NLC were formalin fixed (4%, 10 min, RT), permeabilized (0.1% saponin, 10 min, RT), non-specific binding blocked with a goat serum and then stained with an anti-p65 (ON, 4 °C), followed by an anti-mouse AlexaFluor488 (1 hour, 4 °C) (ThermoFisher Scientific). Immunofluorescence images were acquired using a TCS SP5 laser scanning confocal microscope equipped with 4 lasers, a 63x/1.4 objective lens (Leica Microsystems, Milan, Italy) and the LAS AF version Lite 2.4 software (Leica Microsystems) and processed with Adobe Photoshop (Adobe Systems). Nuclear mean pixel intensities of p65 or % of nuclear p65 (calculated as number of nuclear positive cells over total number of cells) were analyzed using the ImageJ software. In indicated experiments, activation of p65 was induced in NLC by treatment with lipopolysaccharide (LPS, 2 μM, 1 hour, 37 °C) in the presence of vehicle or IT-901 (10 μM).

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted using RNeasy Plus Mini kit (Qiagen, Milan, Italy) and converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was performed using the 7900 HT Fast Real Time PCR System (SDS 2.3 software). Primers for *ATP5A1* (Hs00900735_m1), *SCO2* (HS00192979_m1), *CAT* (HS00156308_m1), *VCAM1* (Hs01003372_m1), *ICAM1* (Hs00164932_m1), *ITGA4* (Hs00168433_m1), *CD274* (Hs01125301_m1), *CD86* (Hs01567026_m1), *ACTB* (Hs99999903 _m1), *B2M* (Hs00984230_m1) were from Life Technologies (ThermoFisher Scientific). Reactions were done in triplicate from the same cDNA reaction (technical replicates). The data were analyzed with the $2^{-\Delta\Delta Ct}$ method, to calculate the relative expression of the gene under analysis. For each gene, expression levels were computed as the difference (ΔCT) between the target gene CT and housekeeping gene CT. Values were normalized over those of Control RNA (Life Technologies, Thermo Scientific) (ΔΔCT), added in each experiment for calibration purposes, and expressed in linear or logarithmic scale.

Generation of lentiviral particles and p65 subunit silencing

Lentiviral particles containing plasmids coding for GFP-shRNA Scramble (shScr) or GFP-shRNA p65 (shp65) (4 different sequences; Origene, Rockville, MD) were generated by transfection of 293T cells with the Effectene kit (Qiagen). After 48 hours medium containing lentiviral particles was removed, centrifuged to pellet any cell debris, followed by ultracentrifugation (28,000 rpm, 2 hours, 4 °C) to concentrate the virus. The pellet, containing concentrated virus, was re-suspended in RPMI without supplements and stored at -80°C.

200,000 Mec-1 or OSU-CLL cells were plated and infected with shScr or shp65 lentiviral particles, monitoring for GFP expression. After few days post-infection, cells were sorted with a FACSAria (BD Biosciences) and an amount used for RNA extraction, while the remaining part was lysed to check p65 expression by western blot. Unsorted infected cells were also tested for apoptosis by flow cytometry, as described above, analyzing the Annexin V/PI staining only on the GFP^+ population.

Immunohistochemistry analyses

Mice organs were formalin-fixed and paraffin-embedded. Sections were deparaffinized and endogenous peroxidase activity blocked with a 6% of hydrogen peroxide solution. Epitope retrieval was performed in 10 mM citrate buffer pH 6 (40 min, 98 °C). Primary antibodies used for immunohistochemical staining were anti-CD20 (clone L26, #NCL-L-CD20-L26, 1:50, Novocastra) and -NF-kB/p65 (clone F-6, Santa Cruz Biotechnology). Anti-mouse HRP-conjugated antibody and 3,3'-diaminobenzidine (EnVision™ System, Dako) were used to visualize the reaction. Slides were analyzed using an AXIO Lab.A1 microscope (Zeiss), equipped with a Canon EOS600D reflex camera and the images were acquired using the ZoomBrowserEX software (Canon). Quantification of brown signal was measured with the ImageJ software.

Statistical analyses

Statistical analyses were performed with GraphPad v6.07 (GraphPad software Inc, La Jolla, CA), significance calculated using the Mann-Whitney or Wilcoxon matched-pairs test and data presented as box plots or histograms. For apoptosis, the mean value was reported in the text with the standard deviation. Kaplan-Meier survival curves were obtained using this software. $P \leq 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***); $P > 0.0001$ (****).

References

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Supplementary Figure legends

Supplementary Figure S1. IT-901 inhibits the c-Rel subunit of NF-kB. (A) DNA binding activity of the c-Rel subunit studied using an ELISA kit, applying the same amount of nuclear extracts of primary CLL cells co-cultured over a stromal layer (HS-5) and exposed to vehicle (NT) or IT-901 at the indicated doses. **(B)** DNA binding activity of c-Rel in 2 different cell line models of CLL, the Mec-1 and OSU-CLL.

OD: optical density

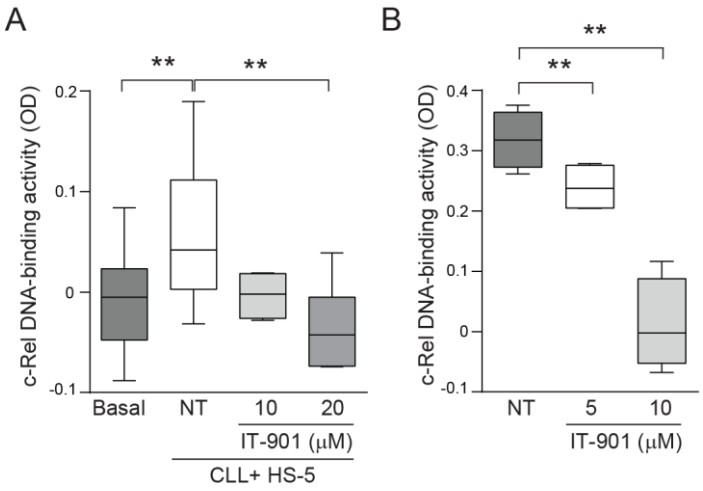
Supplementary Figure S2. IT-901 induces alteration in mitochondrial integrity and cellular metabolism in CLL cell lines. (A) Representative plots and cumulative data of mitochondrial reactive oxygen species (mROS) production (n=9) in CLL cell lines. mROS are represented as fold change (FC) over the vehicle. **(B)** mRNA expression level of catalase in CLL cell lines after exposure for 6h to the indicated doses of IT-901. **(C-D)** Representative plots and cumulative data of inner mitochondrial membrane depolarization, measured using 2 different probes (JC-1 in c and TMRM in d), in CLL cell lines (n=7) exposed to vehicle (NT) or increasing doses of IT-901 for 6h. CCCP was used as positive control. **(E)** Representative mitochondrial metabolic profile (OCR; pmoles/min) obtained by dynamic measurement with the Seahorse platform in Mec-1 cells treated with vehicle (red line), 2.5 μM (green line) or 5 μM (blue line) of IT-901 for 6h. Maximal respiration (calculated as: OCR after FCCP injection - late OCR measurement after RT/AA addition) and ATP production (calculated as: last rate measurement of OCR before Oligo injection – minimum rate measurement after Oligo injection) in CLL cell lines (n=6). **(F)** Box plots reporting mRNA expression levels of genes related to mitochondrial respiration (*ATP5A1, SCO2*).

CCCP: carbonyl cyanide m-chlorophenyl hydrazone; TMRM: tetramethylrodamine-methyl ester; Oligo: Oligomycin; FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone; RT/AA: rotenone + antimycin A; OCR: oxygen consumption rate.

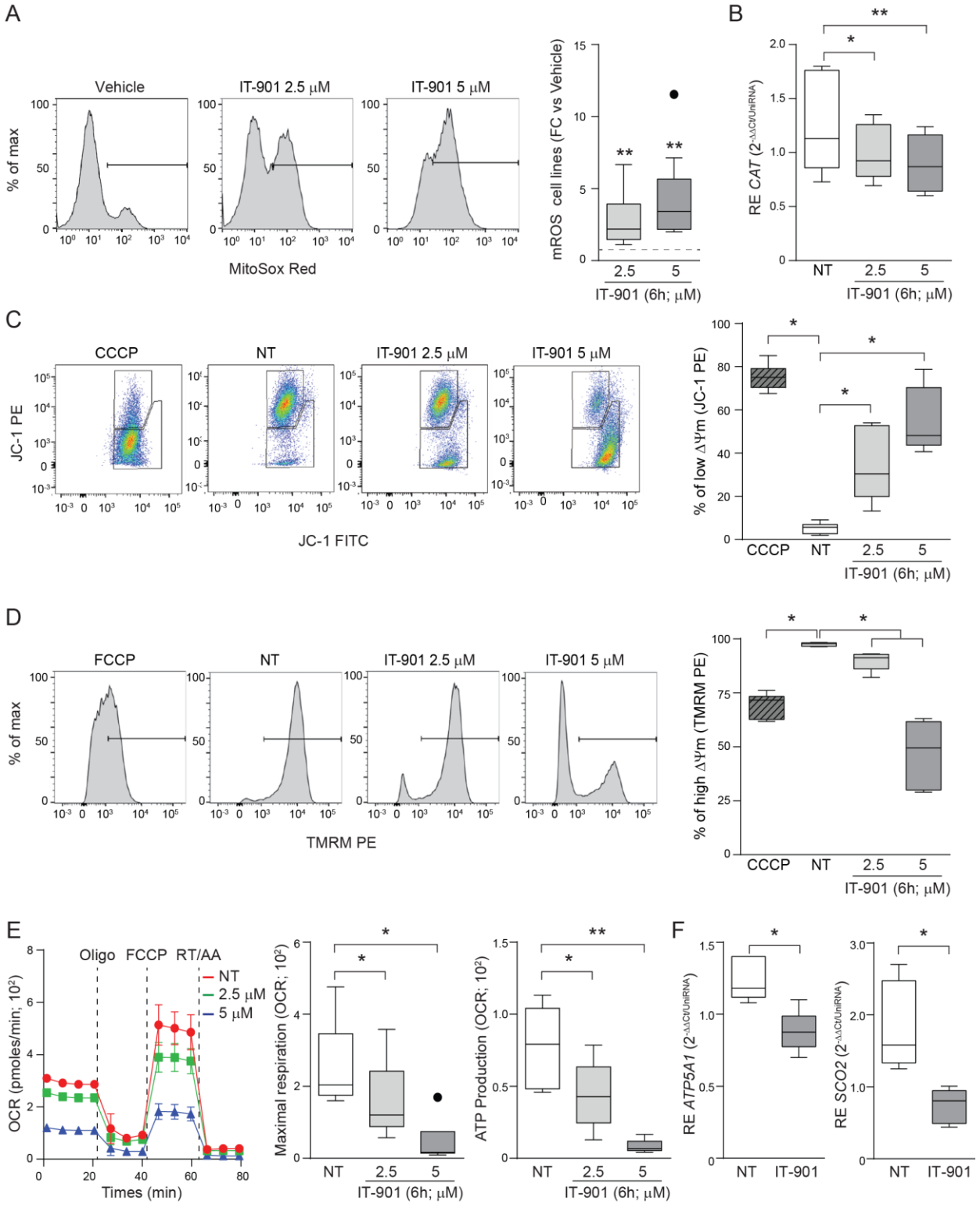
Supplementary Figure S3. IT-901 modulates NF-kB activation in NLC and stromal cells without affecting their viability. (A) Activation of the NF-kB pathway in NLC in basal conditions (NT) or exposed to LPS in the presence of vehicle (LPS) or IT-901 (LPS+IT-901) was checked by confocal microscopy and nuclear localization of the p65 subunit quantified. **(B)** NLC were derived, exposed to vehicle or IT-901 (10 uM, 48h), stained with GIEMSA and counted (left panel). Cumulative apoptosis data of 4 independent experiments performed on HS-5 cells exposed to increasing doses of IT-901 for 24 (middle panel) or 48h (right panel). **(C)** mRNA expression levels of genes involved in OXPHOS (*ATP5A1, SCO2*) in NLC derived from different CLL patients and exposed to vehicle or IT-901 (10 μM; 48h) (left panels). Cumulative data of mROS in HS-5 and mRNA expression levels of catalase (*CAT*) in NLC cells exposed for 6h to vehicle or IT-901 (10 μM). **(D)** Graph showing cumulative data of apoptosis in CLL cells cultured alone (red line), over HS-5 (black line) or autologous NLC (green line). **(E)** NF-kB, pro- and anti-apoptotic proteins and caspase3 expression analyzed by western blot in CLL cells cultured over a stromal layer for 6h in the presence of the indicated doses of IT-901.

FL: full-length; CL: cleaved

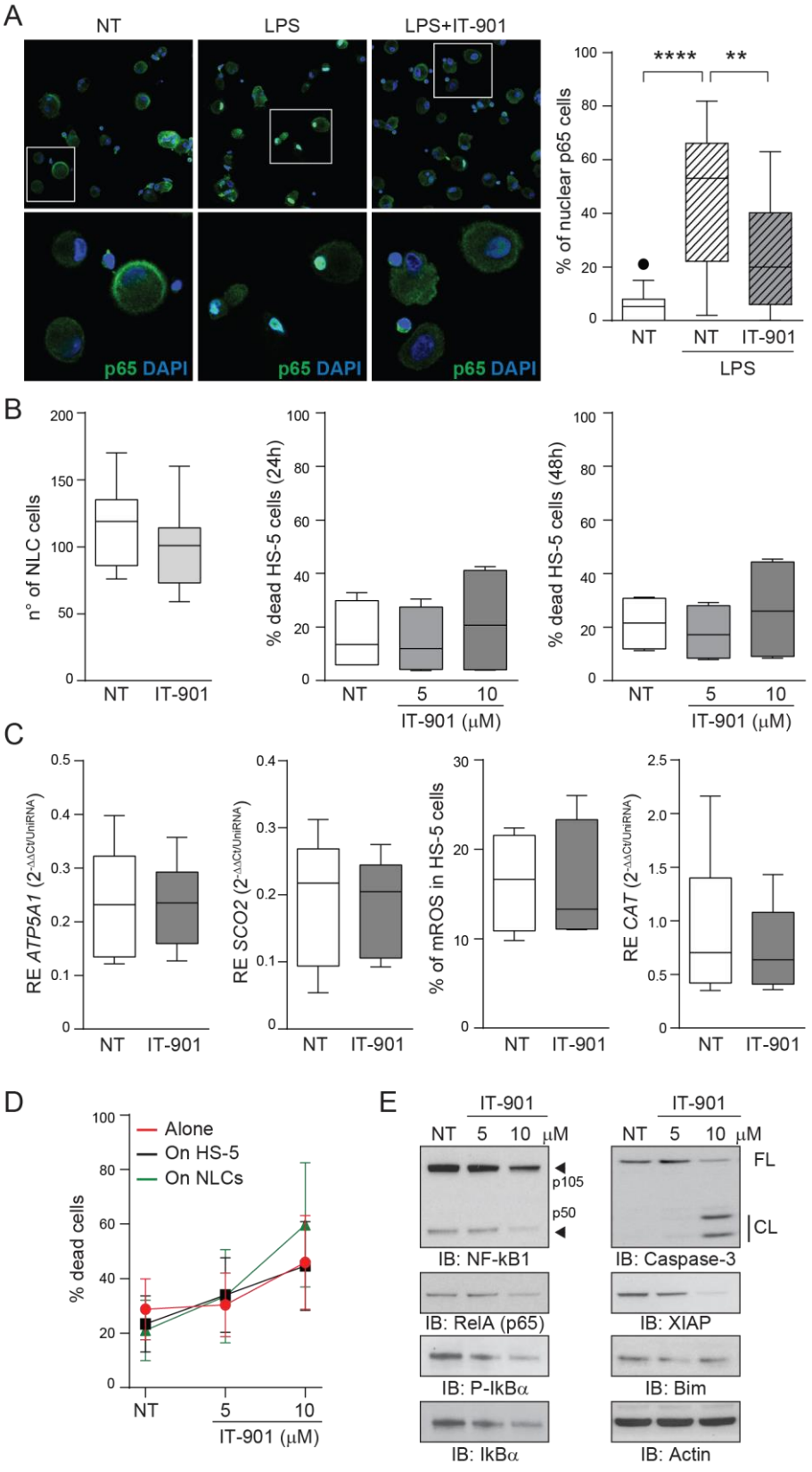
Supplementary Figure S4. IT-901 is effective in a CLL xenograft model and in RS cells. (A) IHC analysis of liver CD20 expression in vehicle- or IT-901-treated mice. **(B)** Expression of the NF-kB complex and Caspase 3 in total lysates obtained from Mec-1 cells purified from kidneys of vehicle- or IT-901-treated mice. **(C)** Western blot analysis of the expression of the NF-kB complex, pro- and anti-apoptotic proteins and Caspase 3 in RS cells cultured over an HS-5 layer and exposed to different IT-901 doses for 6h. **(D)** RNA expression level of the p65 subunit in RS cells obtained from vehicle- or IT-901-treated mice.



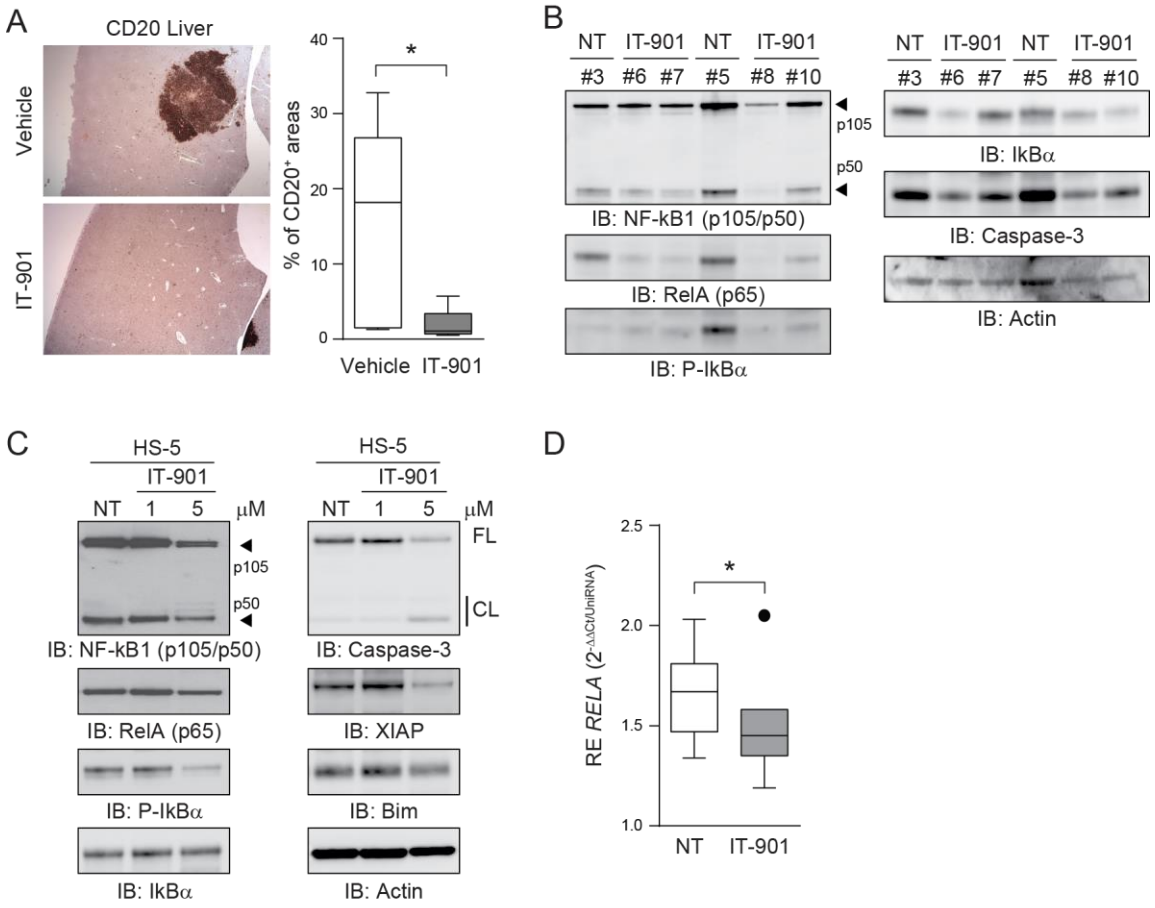
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4