Daratumumab displays *in vitro* and *in vivo* anti-tumor activity in models of B-cell non-Hodgkin lymphoma and improves responses to standard chemo-immunotherapy regimens

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SUPPLEMENTARY INFORMATION

SUPPLEMENTAL METHODS

Cell lines

FL cell lines DOHH-2, RL, SC-1, WSU-FSCCL as well as the Burkitt lmphoma (BL) Daudi cell line and DLBCL cell lines SU-DHL-4 and SU-DHL-6 were obtained from DSMZ (Braunschweig, Germany). MCL cell lines Jeko-1, Mino, REC-1, Z-138 and DLBCL cell line WSU-DLCL2 were obtained from ATCC (LGC Standards, Teddington, UK). HBL-2 was kindly provided by Prof. M Dreyling (University Hospital, Munich, Germany), UPN-1 by Dr. A Turhan (Hôpital Bicêtre et Paul Brousse, Villejuif, France) and Toledo cell line by Dr. MA Piris (Fundación Jiménez Díaz, Madrid, Spain). The RL cell line expressing luciferase (RL-luc) was generated *via* retroviral transduction. Retroviral vectors containing full-length pLHCX-luc were kindly provided by Dr. Bofill-De Ros (IDIBAPS, Barcelona, Spain) (see below method description). RL-GFP cells were produced by the Inserm UMR1037 vector facility (CRCT, Toulouse, France) and provided by JJ Fournié's laboratory (CRCT). Cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 µg/mL penicillin/streptomycin (Life Technologies) and were maintained in a humidified atmosphere at 37ºC containing 5% CO_2. Normocin (100 µg/mL; InvivoGen) was added to the cell line cultures to prevent mycoplasma contamination in cell lines that were routinely tested for mycoplasma infection by PCR. The identity of all cell lines was verified by using the AmpFISTR identifier kit (Life Technologies).

Retroviral transfection of NHL cells

Supernatant of Phoenix cells containing retroviral particles was used to infect RL cells by centrifugation (2500 rpm for 90 minutes) in the presence of polybrene (Sigma Aldrich). Selection of transfected cells was started 2 days after infection with 200 µg/mL of hygromycin B (Sigma Aldrich). The Luciferase Assay System kit (Promega) was used to confirm expression of the luciferase gene, following the manufacturer's instructions.

Antibody-dependent cellular cytotoxicity

ADCC was performed using a calcein-release assay as described previously (30). Target cells were exposed to 10-fold serial dilutions of either isotype control (CNTO 3930, spontaneous release) or daratumumab (range, 1 to 0.0001 µg/mL) in RPMI-1640 in the presence of healthy donor PBMC at E:T ratio of 50:1 for 4 hours. 10% Triton was used to determine maximal release. The percentage of cellular cytotoxicity was calculated using the following formula:

$$\%\ specific\ lysis\ = 100 \times \frac{experimental\ release(RFU) - spontaneous\ release\ (RFU)}{maximal\ release(RFU) - spontaneous\ release\ (RFU)}$$

Antibody-dependent cellular phagocytosis

ADCP was carried out with macrophages generated from monocytes isolated from BM of the hind legs of female SCID mice (C.B-17/Icr-Prkdc^{scid}/Crl; Janvier Labs), by flushing the femurs. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin, and 50 U/ml M-CSF (Cell Guidance), and the culture medium was renewed every 3 days. On day 7, mϕ were detached with 0.1% trypsin-EDTA and characterized by flow cytometry (CD11b^{+}, F4/80^{+}) (mouse antibodies obtained from eBiosciences and Invitrogen, respectively). The mϕ were seeded at 2.5×10^{5} cells per well into non-tissue cultured treated 24-well plates and allowed to adhere overnight. Target cells were labeled with 0.01µM Calcein-AM and added to the mϕ at an E:T ratio of 1:1 in the presence of a fixed mAb concentration of either isotype control (CNTO 3930) or daratumumab at 1 µg/mL. After 4hr of incubation, the non-phagocytosed target cells were collected.The mϕ were detached with 0.1% trypsin-EDTA, added to the non-phagocytosed target cells and stained for F4/80 expression.The amount of remaining target cells (CD19^{+} calcein^{+} F4/80^{-}) was determined on an Attune acoustic cytometer. The percentage of killed target cells in the presence of daratumumab compared with isotype control was calculated using the following formula:

$$\%\ ADCP = 100 - [100 \times \frac{remaining\ target\ cells\ after\ DARA\ treatment}{remaining\ target\ cells\ \ after\ isotype\ control\ treatment}]$$

Complement-dependent cytotoxicity

Target cells were labeled with 1 µM Calcein-AM (Life Technologies) for 30 min at 37ºC. Afterwards, cells were washed thrice with PBS, plated in triplicate at 1×10^{5} cells/well in 96-well round bottom plates, and preincubated (room temperature {RT}, 15 min) with 10-fold serial dilutions of either isotype control (CNTO 3930) or DARA (range: 10 to 0.01 µg/mL) in RPMI 1640. Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, 10% normal human AB serum was added and incubated for 45 min at 37°C. The plates were centrifuged, supernatants transferred into black plates (Thermo Scientific) and fluorescence measured in a Synergy spectrophotometer (Bio-Tek) (excitation filter: 485 ± 20 nm; band-pass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\%\ specific\ lysis\ = 100 \times \frac{experimental\ release(RFU) - spontaneous\ release\ (RFU)}{maximal\ release(RFU) - spontaneous\ release\ (RFU)}$$

3D lymphoma cultures and Selective Plane Illumination Microscopy (SPIM) analysis

Multicellular aggregates of lymphoma cells were generated by the hanging drop method. RL-GFP cells (2.5x10^5 cells/mL) were prepared in complete medium containing 1% Methocult (STEMCELL Technologies), 40 µL of this suspension were pipetted into each well of the Perfecta3D® hanging drop plates (Sigma-Aldrich). After 72 hours of incubation, spheroids were transferred to 96 well plates and treated for 4, 24 and 48 hours with isotype or daratumumab labeled in red with the Mix-n-Stain™CF™ 555 Antibody Labeling Kit (Sigma-Aldrich), then fixed with 4% paraformaldehyde (Sigma-Aldrich) and analyzed by SPIM. Antibody penetration and diffusion were determined according to the following formulas:

$$\%\ penetration\ = 100 \times \frac{Global\ volume\ 561\ nm}{Real\ volume\ 488\ nm}$$

$$\%\ diffusion\ = 100 \times \frac{Real\ volume\ 561\ nm}{Real\ volume\ 488\ nm}$$

Volumes were measured after imaging on an inverted microscope according to the formula:

$$volume\ = \frac{4}{3} \times \pi \times L\ (longest\ diameter) \times l^2 (shortest\ diameter)$$

CD38 molecules per cell quantification

FL and MCL cell lines were stained with QuantiBRITE™ CD38-PE (BD Biosciences) and the mean fluorescence intensities were assessed in an Attune acoustic focusing cytometer (Life Technologies). The specific Antibody Binding Capacity (sABC) was calculated by interpolation in a linear regression curve obtained by QuantiBRITE™ PE Beads (BD Biosciences).

Systemic mouse models

SCID mice were intravenously (iv) injected with 10x10^6 WSU-FSCCL (FL) or Z-138 cells (MCL), following approval of the protocol by the Animal Testing Ethic committee of the University of Barcelona and Generalitat de Catalunya (Protocol # 9971). One week later, mice were randomly assigned into cohorts of 10 mice per group and received one intraperitoneal injection of 20 mg/kg of daratumumab, or rituximab or isotype control on day 7, and thereafter 10 mg/kg weekly for 3 weeks (20/10/10/10 schedule). Mice were monitored twice weekly and sacrificed if they have lost 15% to 20% of weight and/or showed signs of disease. The presence of tumor cells was evaluated first macroscopically and then by flow cytometry. Cells from infiltrated organs were obtained by tissue homogenization. BM cells were obtained after flushing the femoral and tibia bones with RPMI-1640 media. These samples were filtered

through 70 μm nylon sieves (BD Falcon). Erythrocytes were lysed using ACK buffer (Quality Biological Inc.), followed by staining with huCD45/CD19/CD10 antibodies, for the WSU-FSCCL model; or huCD45/CD19 antibodies for the Z-138 model, and analysis by flow cytometer under constant flow rate in an Attune acoustic focusing cytometer (Life Technologies).

In vivo daratumumab combinations in MCL and FL subcutaneous mouse models

SCID mice were subcutaneously (sc) injected with $10x10^6$ RL-luc cells in the FL model or $10x10^6$ REC-1 cells in the MCL model. Mice were randomly assigned into cohorts of 6-8 mice and treated once a week ip with the isotype control, daratumumab, or rituximab, starting when tumors were palpable (day 7) following the schedule (20/10/10 mg/kg in REC-1 and 20/10/10/10 mg/kg in RL-luc). The chemotherapy regimen CHOP was given as an initial single dose with the first dose of antibodies (cyclophosphamide: 25mg/kg; doxorubicin: 2.5mg/kg; vincristine: 0.4mg/kg prednisone: 0.15mg/kg). Tumor volume was measured twice per week with external calipers and calculated using the standard formula: $s^2 \times l \times 0.5$, where (s) is the shortest diameter of the tumor and (l) the longest. As the RL cell line expressed the luciferase gene, sequential bioluminescence images were captured after injecting 150 mg/kg XenoLight D-Luciferin (Perkin Elmer), and measured radiance by Wasabi software (Hamamatsu Photonics). Animals were sacrificed after one month according to institutional guidelines, and tumors were collected and weighed. Tumor samples were formalin-fixed and embedded in paraffin. When indicated, tissue sections were stained with H&E, phosho-histone H3 (pH3) (Epitomics) or CD31 (Santa Cruz Biotechnology) followed by evaluation on an Olympus DP70 microscope using Cell B Basic Imaging Software (Olympus).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Correlation between the number of CD38 molecules per cell and effector mediated cell death. Number of surface antibodies bound per cell (sABC) of CD38 was quantified in MCL, FL and DLBCL cell lines, including Daudi as a positive control, and plotted for correlation with (A) ADCC and (B) ADCP induction by DARA at 0.1 μg/mL and 1 μg/mL, respectively. Statistical significance was assessed by Spearman test.

Figure S2. Daratumumab decreases CD38 surface expression in the presence of macrophages. ADCP was performed in representative MCL, FL and DLBCL cell lines. At the end point (4hr) CD38 surface expression was evaluated in the non-phagocytosed cells using an antibody compatible with daratumumab (HuMax-003 FITC) provided by Janssen. *p* values were calculated using two-way ANOVA.

Figure S3. Daratumumab exerts a direct effect in 3D lymphoma models. 2500 cells from MCL, FL and DLBCL cells lines were seeded on ultra-low attachment plates (ULA) at day 0 and daratumumab (10μg/mL) was added at 3 day for 48hr. Images were captured in a Cytation 1 Imaging system (Biotek) using x4 magnification and Hoechst counterstained (A). Spheroids were measured using Gen5 software and the was volume estimated by the formula (B):

$$volume \ = \frac{4}{3} \times \pi \times L\ (longest\ diameter) \times l^2 (shortest\ diameter)$$

Afterwards, these spheroids were manually disaggregated and cell count was evaluated by flow cytometry (C).

Figure S4. Tumor infiltration in systemic xenograft models of FL and MCL. (A-B) Tumor cells infiltrating the brain, BM, spleen and lungs from the Z138 model were recovered at the endpoint, stained with huCD45/CD19 and counted by flow cytometry. Representative density plots for one mouse of each cohort are shown. (C and D) Total number of huCD45$^+$/CD19$^+$/CD10$^+$ cells from the WSU-FSCCL model were recovered from the brain, spleen and BM of the different treatments and enumerated by flow cytometry. Representative density plots for one mouse of each cohort are shown. Statistical differences between groups were assessed by unpaired *t*-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). CT = isotype control

Figure S5. Daratumumab combined with R-CHOP in tFL. The experimental set up was the one described in figure 5 C-D. (A) Bioluminescence images were captured at endpoint. Representative images of 2 mice from each group are shown as an example of tumor burden. (B) Bioluminescent signal was quantified, and photon emission is represented as the mean of photons/s in 2 mice from each group. (C) IHC staining of H&E, pH3 and CD31 were assessed in representative tumors from each treatment (magnification, 200x).

Figure S6. CD38 expression of DLBCL patient derived xenograft. CD38 expression was assessed by IHC in a tissue section of ST1361 DLBCL patient biopsy

Figure S1

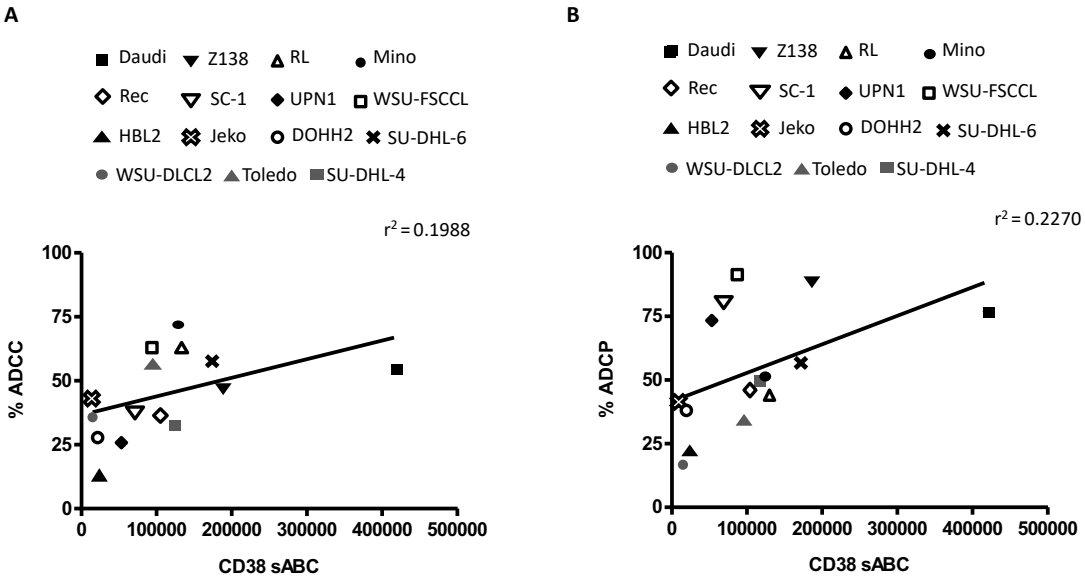
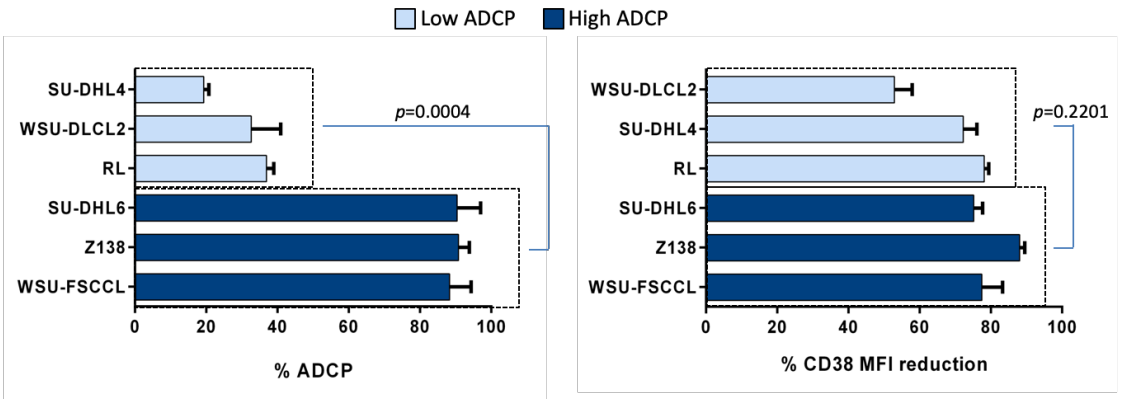


Figure S2



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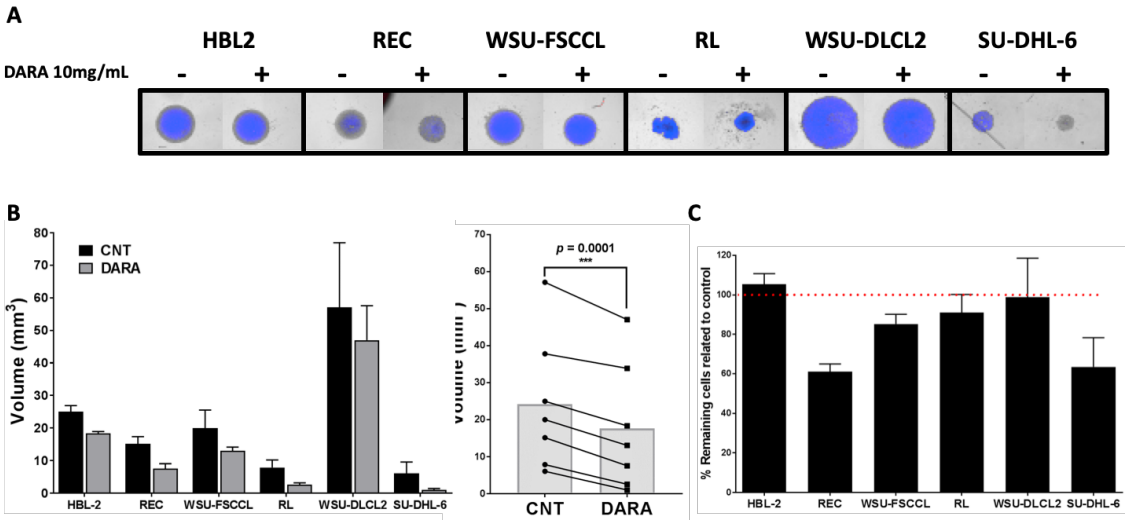

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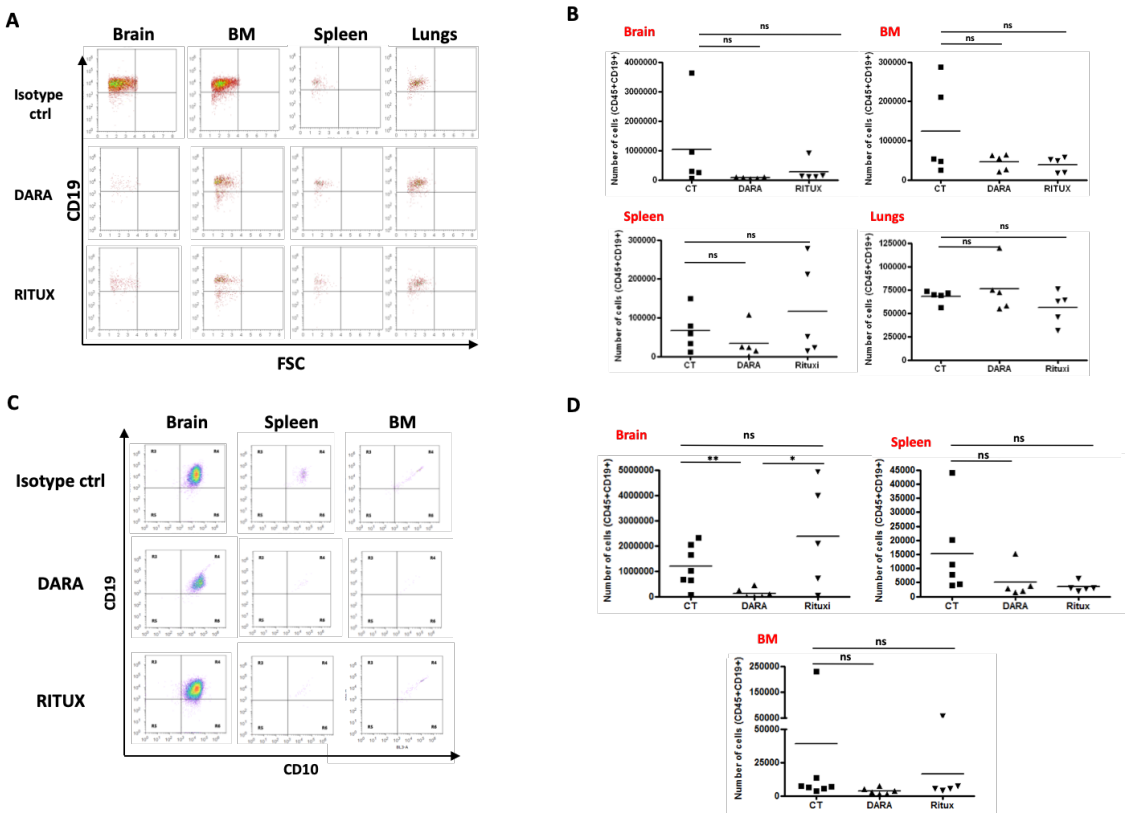

Figure S5

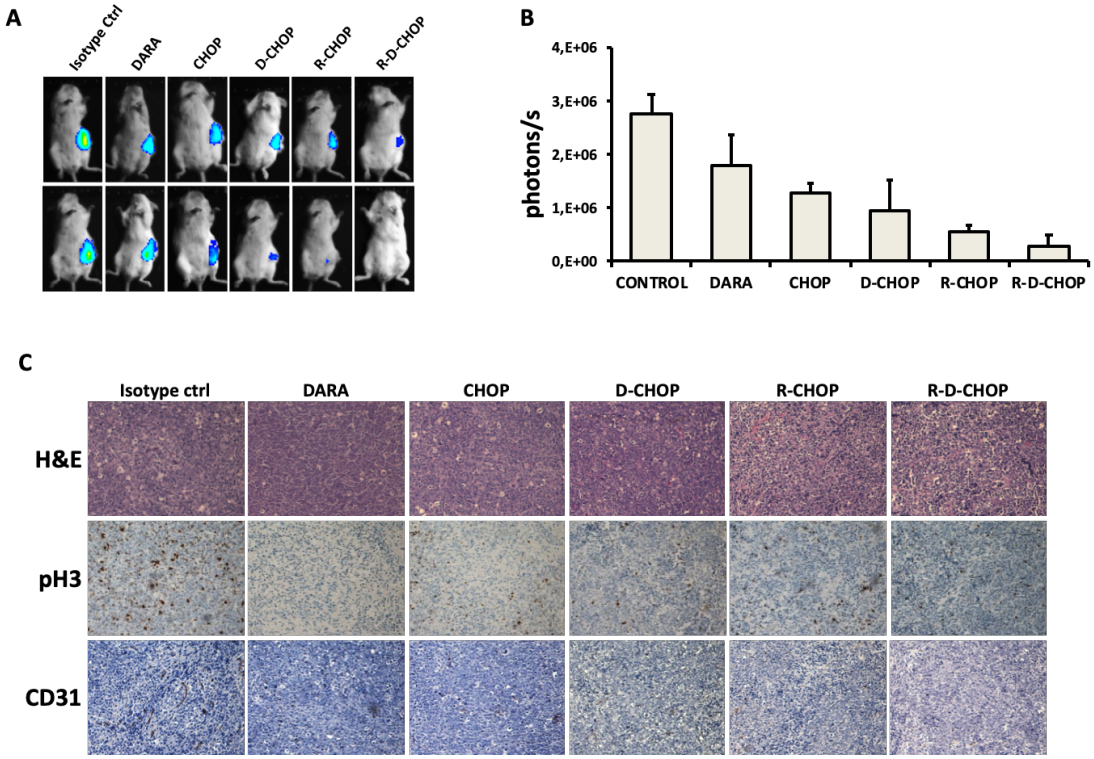

Figure S6