Figure S1

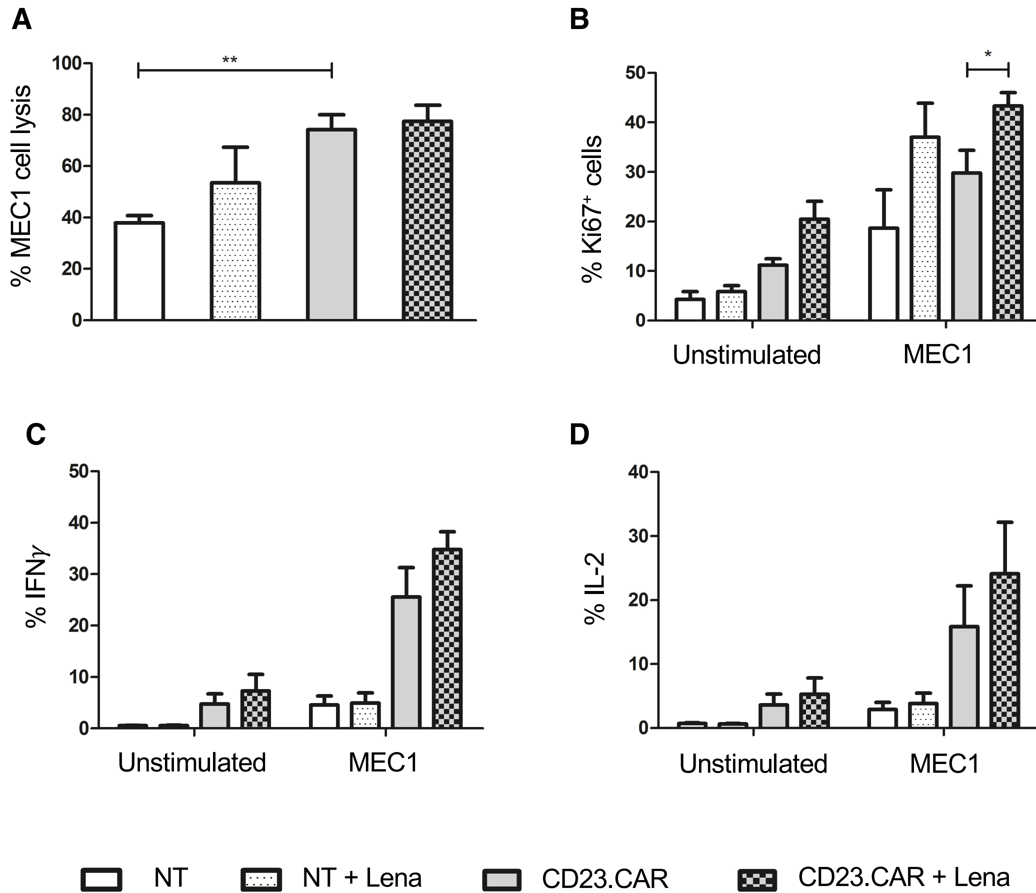

Figure S1. Lenalidomide exerts a costimulatory effect on CD23.CAR+ T cells against MEC1 cells.

(**A-D**) *In vitro* functional characterization of NT and CD23.CAR+ T cells (untreated control or pretreated with lenalidomide) (n=4). The data represent means ± SEM, and unpaired *t*-test was used to compare NT and CD23.CAR+ T cells. (**A**) Short-term cytotoxic assay, E:T ratio, 5:1. **p< 0.01 (**B**) Intracellular staining for Ki67 after 72h. E:T, 1:1. (**C-D**) Intracellular staining for IFN-γ and IL-2 after 5h. E:T, 1:3. The data represent means ± SEM, and unpaired *t*-test was used to compare NT and CD23.CAR+ T cells.

Figure S2

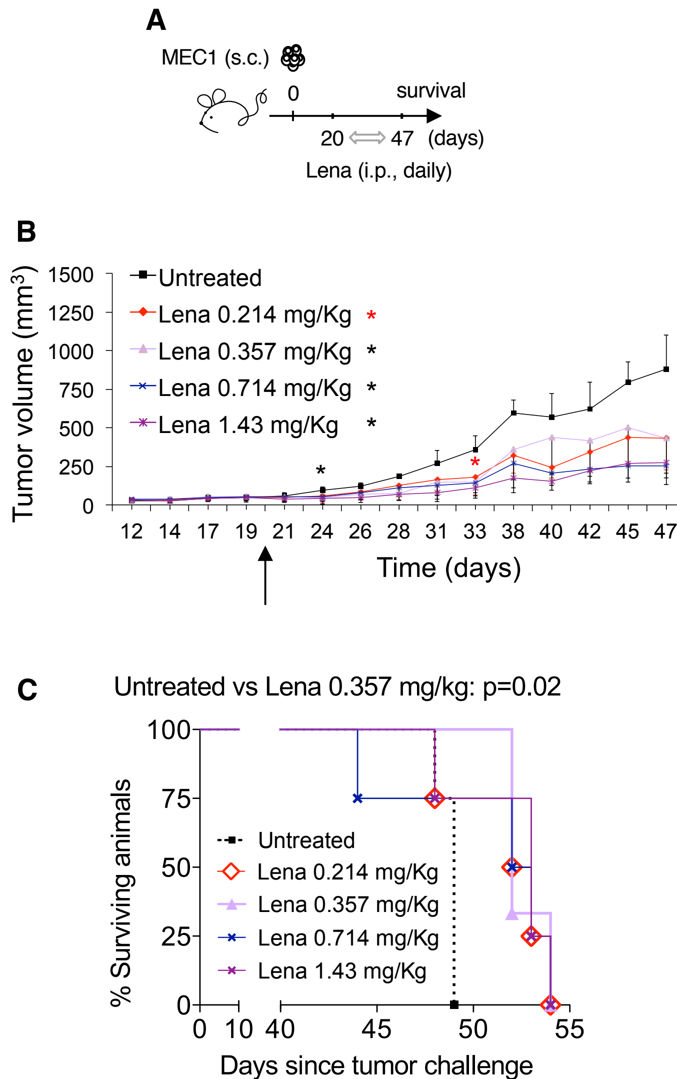

Figure S2. Anti-leukemic effect and survival benefit of lenalidomide in CLL xenotransplanted mice. (**A-C**) Rag2$^{-/-}\gamma_c^{-/-}$ female mice received in the left flank a subcutaneous transplant of MEC1 cells (10×10^6). Twenty days later, when the tumors had reached a mean volume of 95 mm^3, lenalidomide was injected daily (arrow) following the dose schedule used in human clinical trials. Mice were randomly assigned to one of the following intraperitoneal treatments (4 mice/group): untreated (black squares), lenalidomide 15mg/day (0.214 mg/kg, red rhombi); lenalidomide 25mg/day (0.357mg/kg, violet triangles); lenalidomide 50mg/day (0.714 mg/kg, blue crosses); lenalidomide 100mg/day (1.43 mg/kg, purple stars). Each treatment was repeated daily from day 20 to day 47 and animals were monitored for tumor growth, by caliper measurements of perpendicular tumor diameters. Animals were killed when the tumor volume reached 1000 mm^3. Measurements were stopped when 75% of originally treated mice were still surviving. (**B**) Tumor volumes are shown. *Statistically significant differences were calculated using the Student *t*-test: *$P < 0.05$. Black asterisk refers to Lena 0.357mg/kg, 0.714 mg/kg and 1.43 mg/kg compared to untreated control. Red asterisk refers to Lena 0.214 mg/Kg and untreated control comparison. Data are from one representative experiment of two. These two experiments were monitored for survival. (**C**) Kaplan-Meier survival curve is shown, statistical analysis was performed using the log-rank test. Data are from one representative experiment of two.

Figure S3

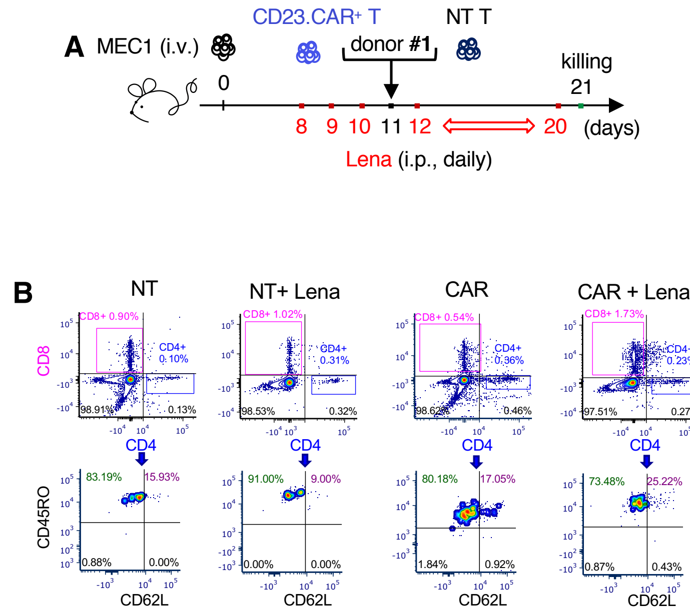

Figure S3. CD23.CAR T cells from patient with CLL persist in the presence of lenalidomide in vivo. (**A-B**) Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted i.v. with MEC1 cells on day 11 of the leukemic challenge were left untreated (Unt, black circles), injected with lenalidomide (Lena) as monotherapy (red rhombi), or adoptively transferred with NT T cells (empty circles), NT T cells with lenalidomide (black triangles), CD23.CAR$^+$ T cells (blue rhombi), CD23.CAR$^+$ T cells with lenalidomide (empty red rhombi). Mice received 0.214 mg/kg of intraperitoneal lenalidomide daily starting at day 8, except for the day of the adoptive transfer. NT and CD23.CAR$^+$ T lymphocytes were obtained from CLL donor #1. At day 23 after the transplantation, mice were evaluated by flow cytometry analysis for the presence of human lymphocytes in the lymphoid tissues. (**B**) Representative flow cytometry plots of human CD8$^+$ and CD4$^+$ T lymphocytes expressing CD45RO CD62L in the BM of mice adoptively transferred with NT or CD23.CAR$^+$ T cells (alone or in combination with lenalidomide) from CLL patient #1.

Figure S4

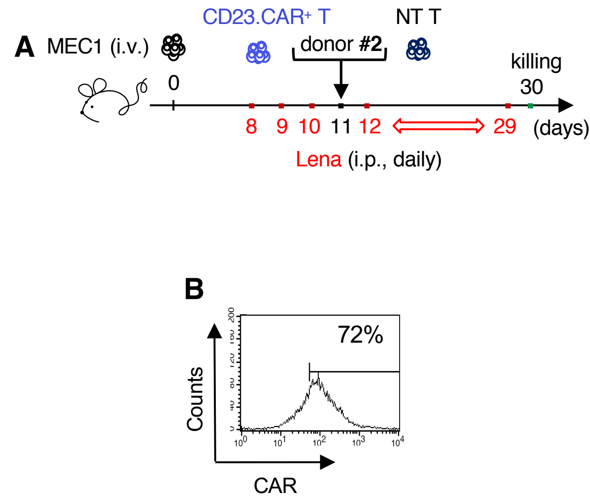

Figure S4. Lenalidomide sustains the *in vivo* persistence of CD23.CAR$^+$ T lymphocytes from CLL patients. (**A-B**) Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted i.v. with MEC1 cells were injected with NT T cells with lenalidomide or CD23.CAR$^+$ T cells with lenalidomide. Mice received 0.214 mg/kg of intraperitoneal lenalidomide daily starting at day 8, except for the day of the adoptive transfer (day 11). NT and CD23.CAR$^+$ T lymphocytes were obtained from CLL donor #2. At day 30 after the transplantation, mice were evaluated for the presence of CD23.CAR T cells. (**B**) Expression of anti-CD23.CAR on the surface of T lymphocytes purified from the BM of xenotransplanted mice (day 30) treated with CD23.CAR$^+$ T cells in combination with lenalidomide evaluated by flow cytometry with a Cy5-conjugated-anti-human-Fc antibody (CAR).

Figure S5

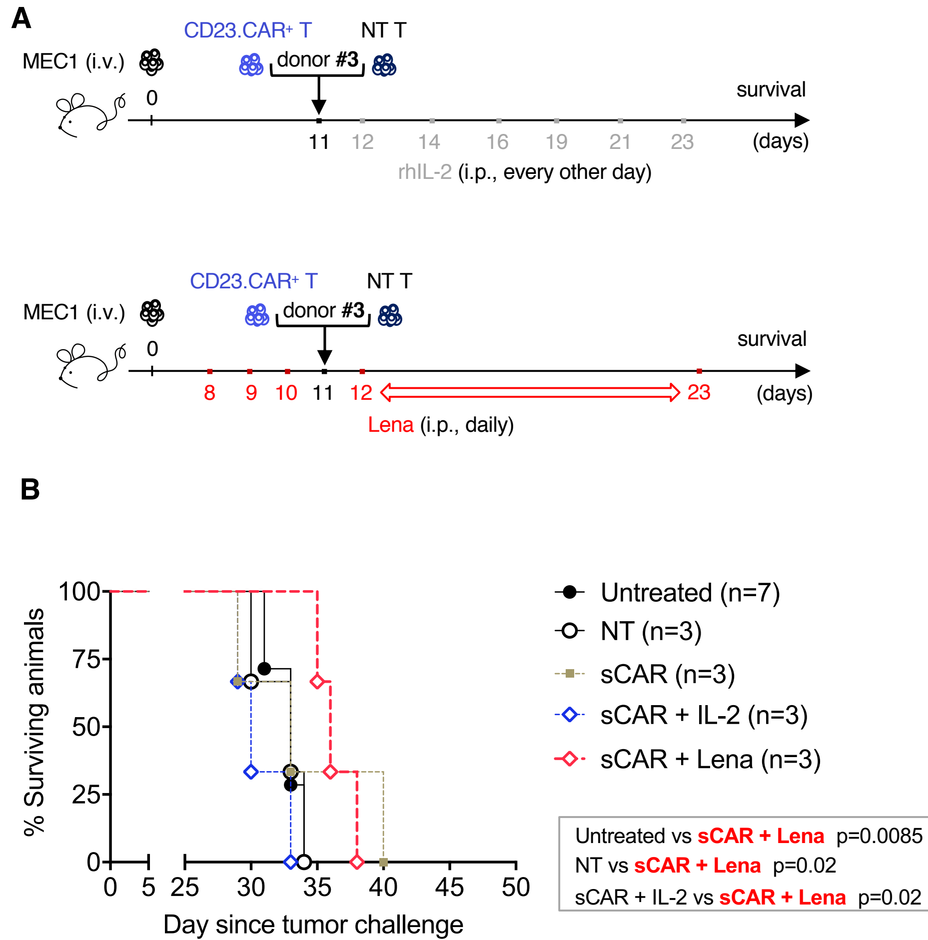

Figure S5. Lenalidomide in combination with CD23.CAR$^+$ T lymphocytes from CLL patients delays disease progression. (**A-B**) Rag2$^{-/-}\gamma_c^{-/-}$ mice who received MEC1 cells intravenously on day 0 were left untreated (black circles) or given NT T lymphocytes (days 11); CD23.CAR$^+$ T lymphocytes (at days 11) alone (golden square) or with rhIL-2 every other day starting at day 12 (six administrations, empty blue rhombi); or CD23.CAR$^+$ T lymphocytes (at day 11) with daily lenalidomide from day 8 (empty red rhombi) and monitored for survival. NT and CD23.CAR$^+$ T lymphocytes were from CLL donor #3. sNT and sCAR refers to single adoptive transfer (days 11). (**B**) Kaplan-Meier survival curve is represented; statistical analysis was performed using Log-Rank test and is indicated in Figure.

1 **Supplemental methods**

2

3 **Cells and Reagents**

4 MEC1 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal
5 bovine serum and 15 µg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA). MEC1 cells have
6 been obtained in 2014 from DSMZ, they were cultured for 1-2 weeks and frozen at low passages
7 (5-10). For *in vitro* and *in vivo* experiments MEC1 cells were thawed, cultured for 1-2 weeks and
8 used at 15-20 passages. The cells regularly tested negative for *Mycoplasma* contamination (PCR
9 Mycoplasma Detection Kit, Applied Biological materials Inc., Richmond, BC, Canada) and were
10 authenticated in the past year.

11 For *in vitro* studies, lenalidomide (pure powder) was dissolved in dimethyl sulfoxide (DMSO,
12 Sigma-Aldrich) to create 10 mM stock solutions that were maintained at -20 °C for no longer than
13 1 week. For *in vivo* studies, the stock solutions were diluted in sterile 0.5% normal saline to a final
14 concentration of 1mg/ml and stored at 4 °C for the duration of the experiment. The final
15 concentration of DMSO in all experiments was < 0.01% (1).

16 **Immunophenotyping**

17 For *in vitro* studies, human cells were stained with the following antibodies: FITC Anti-Human
18 CD19, HIB19 clone; PE Anti-Human CD23, M-L233 clone; FITC Anti-Human CD45RO, UCHL1
19 clone, PE Anti-Human CD62L, DREG-56 clone; FITC Anti-Human CD8 from BD; PE Anti-
20 Human CD4, SK3 clone. All the antibodies were obtained from BD Biosciences (San Jose, CA,
21 USA). Anti-Fc-γCy5 antibody was obtained from Jackson ImmunoResearch (West Grove, PA,
22 USA)(2).

23 Cell death and apoptosis were detected using the GFP-Certified™ Apoptosis/Necrosis Detection
24 Kit (Enzo Life Sciences Inc., Farmingdale, NY, USA), according to the manufacturer's
25 instructions. T cells and MEC1 cells were labeled with FITC- and PE- CellTracker™ (Invitrogen,
26 Carlsband, CA, USA).

27 Samples were acquired using the FACS Canto II flow cytometer (BD Biosciences) and data were
28 analyzed using BD FACS DIVA software version 6.1.3 (BD Biosciences). T cells have been
29 identified as follows: naïve CD45RA$^+$ CD45RO$^-$CD62L$^+$ T_N cells, effector memory CD45RA$^-$
30 CD45RO$^+$ CD62L$^-$ T_{EM}, central memory CD45RA$^-$ CD45RO$^+$ CD62L$^+$ T_{CM}, terminally
31 differentiated CD45RA$^+$ CD45RO$^-$ CD62L$^-$ T_{EMRA}.

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33 **Proliferation assay**

34 NT and CD23.CAR^+ T cells, both untreated control and those pretreated for 48 hours with 1 μM
35 lenalidomide, were co-cultured with irradiated target MEC1 cells (E:T ratio, 1:1). After 72 hours
36 of co-culture, cells were stained for CD3 and CAR, and Ki67 (clone B56; BD Biosciences) was
37 used to assess NT and CD23.CAR^+ T cell proliferation via flow cytometry.

38 **Intracellular cytokine staining**

39 NT and CD23.CAR^+ T cells, both untreated control and those pretreated for 48 hours 1 μM
40 lenalidomide, were co-cultured with MEC1 cells (E:T ratio, 1:3). After 2.5 hours of co-culture,
41 GolgiStop (BD Biosciences) was added. After an additional 2.5 hours of co-culture, the cells were
42 stained for CD3 and CAR. Interferon γ (IFNγ; clone B27, BD Biosciences) and IL-2 (clone MQ1-
43 17H129; BD Biosciences) were used for intracellular staining.

44 **Nanostring**

45 CAR-T Characterization Panel (Nanostring) enriched with 16 additional gene-specific probes for
46 genes involved in the "immunological synapse" (ALCAM, CD37, CD53, CD81, DLG1, FN1,
47 FOXP1, CD11a, LGALS3, MYH9, NFATc2, NFATc3, NFATc4, NUMB, SCIMP, VHL) was
48 used following Nanostring nCounter® standard procedures. RNA quality and quantity have been
49 assessed using 2100 Bioanalyzer (Agilent) following manufacturer instructions. 50ng of total RNA
50 for each sample were hybridized at 65°C for 22 hours with a mix of standard and custom probes
51 following manufacturer instructions. Hybridized samples were loaded on Nanostring Automated
52 nCounter® Prep Station with FLEX system for cartridge preparation and further processing. Raw
53 expression data were analysed with nSolver Analysis Software 4.0 (Nanostring).

54 **In vivo studies**

55 Eight-week-old $Rag2^{-/-}\gamma c^{-/-}$ female mice were transplanted either intravenously (i.v.) or
56 subcutaneously (s.c., in the left flank) with 10×10^6 MEC1 cells (3).

57 Mice bearing subcutaneous tumors were given intraperitoneal (i.p.) injections of lenalidomide
58 (0.214, 0.357, 0.714, or 1.43 mg/kg) from day 20 to day 47 and were sacrificed when the mean
59 tumor volume was ≥ 1000 mm^3, according to standard ethical animal guidelines.

60 For combination treatment studies, $Rag2^{-/-}\gamma c^{-/-}$ mice that had received i.v. injections of MEC1 cells
61 were adoptively transferred with 10×10^6 CD23.CAR^+ T cells or NT T cells, obtained from patients
62 with CLL. These T-cell injections were given alone or in combination with i.p. lenalidomide

63 (0.214 mg/kg) or 500U of recombinant human interleukin-2 (rhIL-2) as previously described (4).
64 Depending on the experiment, mice were sacrificed at late-stage of leukemia or kept for survival.
65 **Murine cell preparation and flow cytometry**
66 Peripheral blood (PB), peritoneal exudates (PE), spleens (SP), and femurs were collected from
67 mice, and cells (including human and murine) were isolated. Erythrocytes from bone marrow
68 (BM), PE, SP and PB samples were lysed by incubation in ammonium chloride solution lysis
69 buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.2-7.4) for 5 min at room
70 temperature.
71 For murine cell immunophenotyping, a Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo
72 Fisher Scientific, Waltham, MA, USA) was used first to gate out dead cells. After blocking the
73 fragment crystallizable (Fc) receptors with Fc block (BD Biosciences) for 10 minutes at room
74 temperature, cells from the PB, BM, SP, were stained (15 min. at 4 ºC) with the antibodies listed
75 in Supplemental Table 1. An IntraPrep Permeabilization Reagent Kit (Beckman Coulter, Brea,
76 CA, USA) was used for the intracellular detection of murine IL-6.
77 For human B- and T-cell immunophenotyping, cells from the PB, SP and BM were incubated with
78 LIVE/DEAD fixable Aqua dye. Then, after blocking the Fc receptors, the cells were stained with
79 surface antibodies described in Supplemental Table 2. Gating adjustments were made based on
80 fluorescence-minus-one (FMO) controls. Samples were acquired using a LSRFortessa X-20 (BD
81 Biosciences) and analyzed with FCS Express 6 Flow Cytometry Software (De Novo Software,
82 Glendale, CA, USA). $hCD4^+$ or $hCD8^+$ T cells have been identified as follows: naïve $CD45RO^-$
83 $CD45RA^+CD62L^+$ T_N cells, effector memory $CD45RA^-CD45RO^+CD62L^-$ T_{EM}, central memory
84 $CD45RA^-CD45RO^+CD62L^+$ T_{CM}.
85 In some experiments, phenotype analysis of MEC1 human leukemia cells from xenotransplanted
86 mice was performed with PE-Cy7 mouse anti-human CD19 (J3-119) from Beckman Coulter and
87 mouse PE anti-human CD23 (TU1) from Invitrogen. Cell-surface expression levels were analyzed
88 using a Cytomics FC500 (Beckman Coulter).
89 Absolute cell numbers were obtained by multiplying the percentage of the cells by the total number
90 of splenocytes, and BM cells flushed from 1 mouse femur.
91 **Cytotoxicity assay in xenograft studies**
92 In xenograft studies, BM cells were flushed from mouse femurs and tibiae, and pre-injected human
93 T cells were then isolated from BM cell suspensions using magnetic beads (Miltenyi Biotec,

94 Bergisch Gladbach, Germany). After 12h of in vitro culture without re-stimulation, the cytotoxic
95 activity of NT and CD23.CAR^{+} T cells towards MEC1 target cells was evaluated in a 4-h co-
96 culture assay (E:T ratio, 3:1). Cells were collected, stained with anti-CD23 antibody, and analyzed
97 using flow cytometry as described previously (5).

98 **Nanostring data statistical analysis**

99 Normalized data were log2 transformed and analyzed with R software platform version 4.0.3
100 (https://www.r-project.org/). To support visual data exploration, heatmaps for most relevant genes
101 were generated using the heatmap.2 function from the gplots package, following Nanostring pre-
102 specified Annotations. We employed a Shapiro-Wilk test to verify if for genes from pathway of
103 interests the fold change data between lenalidomide (+L) and untreated (-L) follow a normal
104 distribution in NT and CD23.CAR+ T cells. Accordingly, a paired t-test, or paired nonparametric
105 Mann-Whitney U test was applied to assess the relationship between gene expression and cell type.
106 A box-and-whisker plot (Box plot represents first (lower bound) and third (upper bound) quartiles,
107 whiskers represent 1.5 times the interquartile range) was used to visualize the data.

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