Supplementary Information

Methods

Generation of B2M-Knockout cells

To obtain MHCI-deficient tumor cells, we utilised CRISPR technology to knockout *B2M* (Beta-2-Microglobulin) which is a critical component of MHCI complex for antigen presentation. To generate 4T1 mammary cells with a knockout of *B2M*, gRNAs were designed to bind within either exon 1 or exon 2 of the gene using the CRISPOR algorithm (1). The gRNAs were cloned into the lentiviral expression vector lentiCRISPRv2 using BsmBI.v2 (NEB) insertion sites (2). Some gRNAs were cloned into a modified lentiCRISPRv2 plasmids, containing VQR-mutant or VRER-mutant S.p.Cas9 with altered DNA-binding specificities (3). Plasmids were verified by sequencing. Lentivirus was produced as previously described (4). Briefly, $1x10^6$ HEK293FT cells were plated into a poly-D-lysine-coated 60-mm dish (Corning) The following day, the cells were transfected with 4.8µg of the cloned lentiCRISPRv2 containing the gRNAs, 2.4µg of pMDLg/pRRE (#12251 Addgene, Cambridge, MA), 1.6µg of pRSV-REV (#12553 Addgene), and 0.8µg of phCMV-VSV-G (#8454 Addgene) using calcium phosphate transfection kit (Sigma-Aldrich) in the presence of 25µM chloroquine (Sigma-Aldrich). The medium was changed 16 hours post-transfection, and the virus particles were collected after an additional 28h, by filtering the supernatant through a 0.45µm filter and stored at -80^{o}C. 10,000 4T1 cells suspended in 2ml of RPMI medium supplemented with 10% FBS per condition were plated and allowed to adhere to a 6-well plate (Corning). Four hours post-plating, cells were transduced with 500µl of virus-containing supernatant for 6 hours in 8µg/ml protamine sulphate (Sigma Aldrich). After transduction with either sgRNA-3 or sgRNA-4, cells were cultured for four days and then stained using PE-conjugated anti-mouse H-2Kd antibody (BD Biosciences) and FACS-sorted (BD Aria fusion). FACS-sorted cells were further sub-cultured for at least a week prior to injection. To further ensure that there was no re-emergence of H-2Kd^{+} cells, cells that did not express H-2Kd were negatively selected using anti-PE microbeads (Miltenyi Biotec) (Supplementary Figure 4).

In vivo studies

All in vivo procedures were performed in strict compliance with approved ethical permits by the Swedish board of Agriculture (8525-2020, 6197-2019). Mice were housed in the animal facility with a constant temperature (20 ± 2 °C), constant humidity (50 ± 10%), and constant 12-hour day/night light cycle with food and water provided equilibrium. Any signs of body weight loss were monitored after tumor inoculation throughout. Tumor volume was monitored and measured every other day and calculated according to the standard formula (length × width^2 × 0.52). All the mice were randomly divided to each group, minimizing cage effects as potential confounder. Sample size was chosen to achieve a greater than 95% probability of identifying, by appropriate statistical analysis. Experimental outliers such as unusual tumor growth or necrotic tissue samples were excluded from analysis.

For the 4T1 metastatic mammary model, 0.2x10^6 wild-type or *B2M*-KO 4T1 cells were injected on day 0 into the mammary fat pad of 5-7 week-old female Balb/cAnNCrl mice (SCANBUR) (n=8 per group). Tumors, and spleen were collected on day 18. For FACS analysis, a part of the tumor was processed and digested with tumor dissociation kit (Miltenyi Biotech). After removal of muscles from the bones, single cells from bone marrow were then flushed by a 27G needle and syringe filled with PBS containing 10% FBS.

Female C57BL/6 mice at 7-week-old (Janvier) were used for B16F10, EO771, RMA and RMA-S experiments. 0.1x10^6 B16F10 cells were resuspended in 50µL phosphate buffered saline (PBS) and injected subcutaneously into the right flank. For EO771 model, 1x10^6 cells were injected on day 0 into the mammary fat pad of female mice. For the RMA and RMA-S models, 0.1x10^6 and 1x10^6 cells were injected subcutaneously into the right flank respectively. NK cells were depleted using conventional anti-NK1.1 depletion antibody treatment in vivo. Anti-NK1.1 neutralizing antibody (35mg/kg body weight, Mabtech) was intraperitoneally injected one day before tumor injection and then once per week. In both the B16F10 and RMA models, mice were sacrificed using a lethal dose of CO_2 after 18 days post tumor implantation.

For NK cell infusion, NK cells were isolated from a pool of four healthy murine spleens via negative selection based on manufacturer's protocol (Mouse NK cell isolation kit, Miltenyi Biotec). Purified NK cells were then co-cultured with either CFSE-labelled RMA or RMA/S cells for 48 hours, supplemented with 300 IU/ml of human IL-2 (Proleukin). NK cells were FACS-sorted (BD Aria Fusion) for purification and then intravenously injected (tail vein) at $0.5x10^6$ cells into healthy C57BL/6 mice. Etanercept (Sigma Aldrich) was administered intraperitoneally at 3mg/kg body weight on day 0,3 and 6. All mice were sacrificed after day 7.

Flow Cytometry

Single-cell suspensions of PBMC and tissue samples were washed with FACS buffer (5% FBS in PBS) before staining with antibody mixes (see Supplementary Table 1). FACS data were acquired using Novocyte Quanteon and analysed using FlowJo (BD) (Gating strategy in supplementary figure 5). For intracellular staining of cytokines, cell suspensions were treated overnight with Golgi-stop and Golgi-Plug (BD Biosciences). Intracellular staining procedures were carried out with BD Cytofix/Cytoperm^{TM} kit (BD Biosciences) with recommended protocol from manufacturer. Annotated pie charts were generated by Pestle and SPICE software developed by National Institutes of Health (NIH).

CFU and Myeloid output assay

$1x10^5$ splenocytes were cultured in 6-well plates with 1ml of MethoCult Media (Stem cell technologies). After 10 days, colonies were counted (minimum area> 10^4 μm^2) on incucyte S3 platform and flow cytometry was thereafter performed on harvested cells.

Statistics

Unless stated otherwise, data were analyzed by Graphpad Prism software by either student *t*-test (2 groups) or one-way ANOVA with multiple comparisons. Results were presented in tukey's boxplots. ns= non-significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

References

1. Haeussler M, Schonig K, Eckert H, Eschstruth A, Mianne J, Renaud JB, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016;17(1):148.
2. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014;11(8):783-4.
3. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015;523(7561):481-5.
4. Sutlu T, Nystrom S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther. 2012;23(10):1090-100.

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Marker	Fluorophore	Catalog#	Vendor	comments
FIXABLE LIVE/DEAD	AMCYAN	L34957	THERMO FISHER SCIENTIFIC	Dead cell marker
FIXABLE LIVE/DEAD	APC-CY7	L34975	THERMO FISHER SCIENTIFIC	Dead cell marker
CD117	BV785	135138	BIOLEGEND	
SCA-1	BV421	108127	BIOLEGEND	
CD127	ALEXA FLUOR 488	135017	BIOLEGEND	
CD34	PE-CY7	128617	BIOLEGEND	
CD16/CD32	APC	156607	BIOLEGEND	
H-2KD	PE	553566	BD BIOSCIENCES	
I-A/I-E	PERCP	107623	BIOLEGEND	
LY6G	PACIFIC BLUE	560603	BD BIOSCIENCES	
LY6C	PE	128007	BIOLEGEND	
TER-119	PE	116207	BIOLEGEND	Lineage Marker
CD45R	PE	561878	BD BIOSCIENCES	Lineage Marker
CD3	PE	553064	BD BIOSCIENCES	Lineage Marker
GR-1	PE	553128	BD BIOSCIENCES	Lineage Marker
CD11B	PE	101207	BIOLEGEND	Lineage Marker
H2Kb	BV421	116525	BIOLEGEND	
IL-4	PERCP/CY5.5	504123	BIOLEGEND	
IL-6	APC	504507	BIOLEGEND	
IL-10	PE-DAZZLE 594	505033	BIOLEGEND	
GM-CSF	PE-CY7	505411	BIOLEGEND	
CD3	PE/Fire700	100272	BIOLEGEND	
CD45	ALEXA FLUOR 700	103128	BIOLEGEND	
CD11B	PE-CY7	25-0112-82	EBIOSCIENCES	
CD11C	FITC	11-0114-82	EBIOSCIENCES	
IFNY	BV785	505837	BIOLEGEND	
TNF-A	BV605	506329	BIOLEGEND	
NK1.1	BV421	108741	BIOLEGEND	

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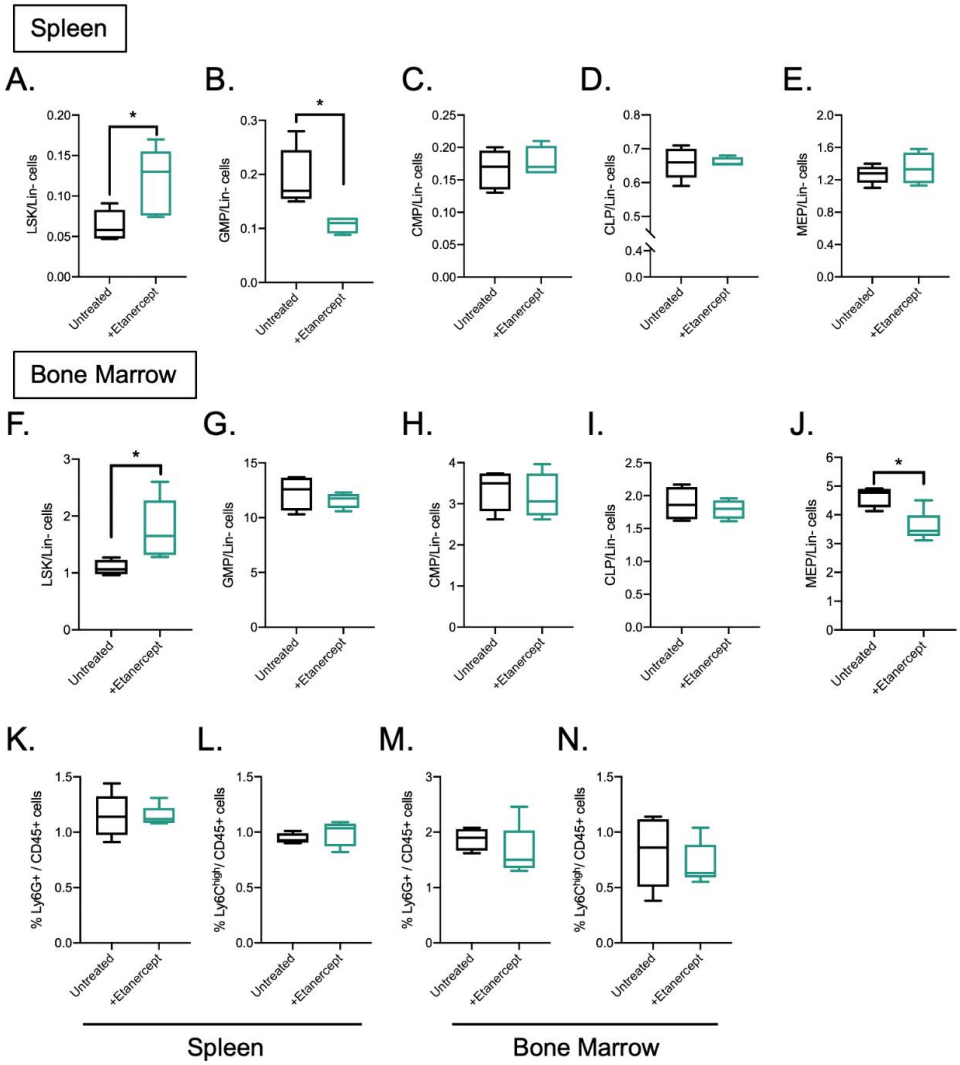
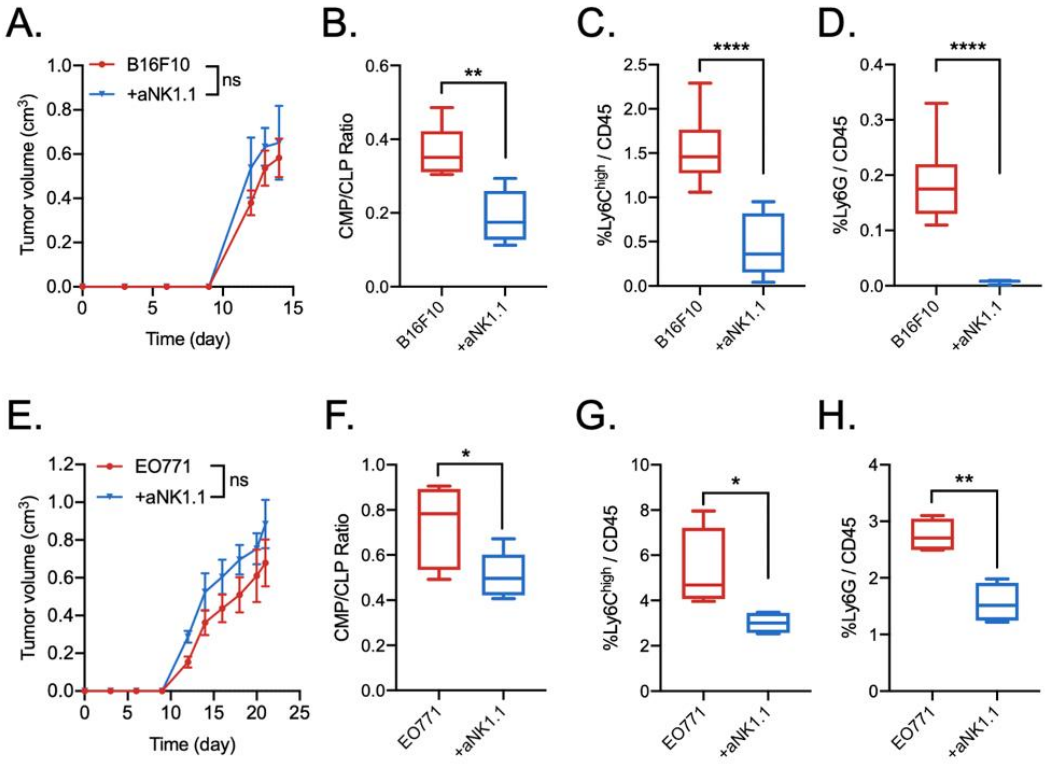
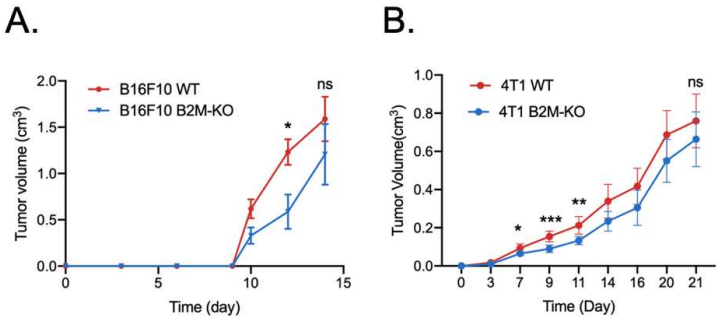


Supplementary Figure 1. Etanercept treatment alone does not influence myelopoiesis in spleen and bone marrow.

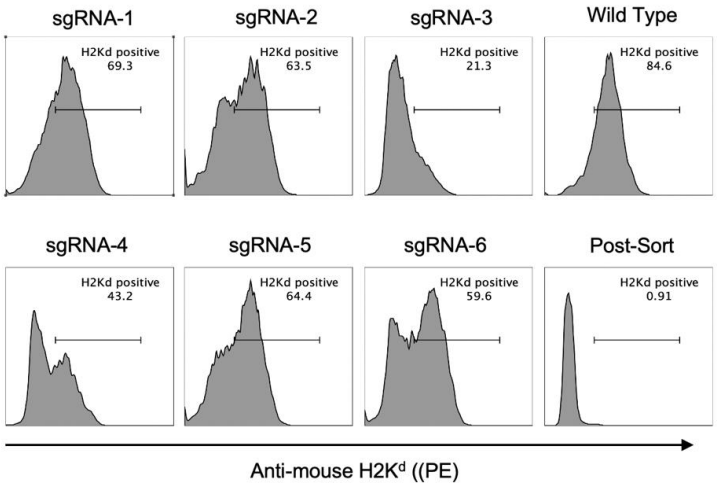
A to E, Frequencies of **(A)** LSK cells, **(B)** GMP, **(C)** CMP, **(D)** CLP and **(E)** MEP over lineage-negative cells in the spleen.
F to J, Frequencies of **(F)** LSK cells, **(G)** GMP, **(H)** CMP, **(I)** CLP and **(J)** MEP over lineage-negative cells in the bone marrow.
K and L, Frequencies of **(K)** Ly6G+ and **(L)** Ly6C^{high} myeloid cells over total CD45+ cells within the spleen.
M and N, Frequencies of **(M)** Ly6G+ and **(N)** Ly6C^{high} myeloid cells over total CD45+ cells within the bone marrow.
A to N, Comparison between untreated C57BL/6 mice (n=5) and Etanercept-treated mice (n=5) were tested for significance using student t-test. Unless indicated * for significance ($p<0.05$) otherwise, all other comparisons are not significantly different between the 2 groups.



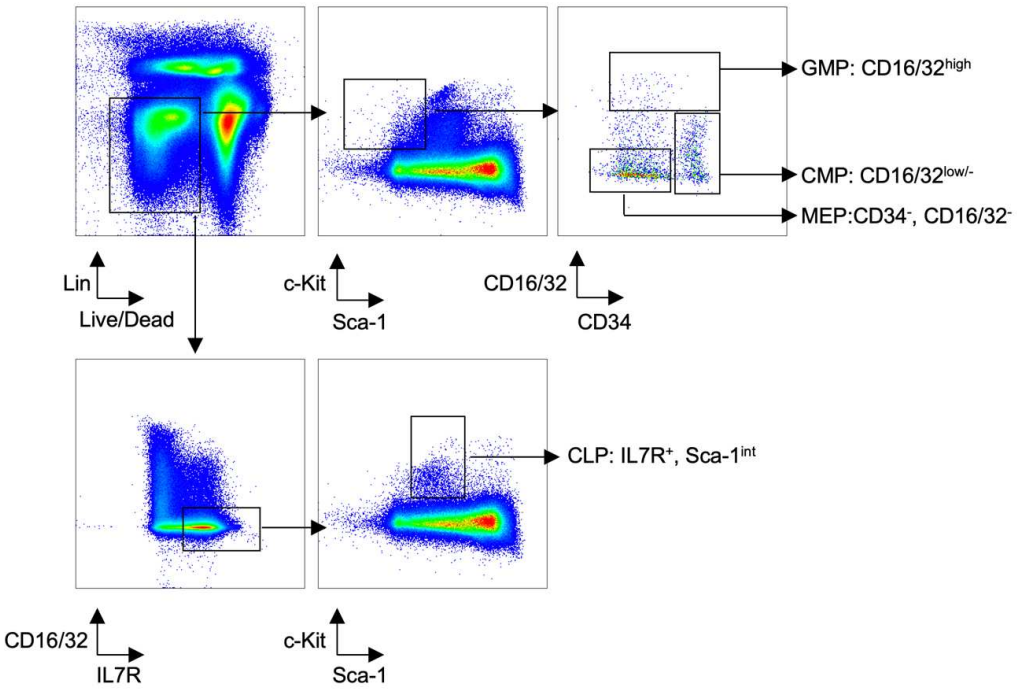
Supplementary Figure 2. NK cell depletion in tumor-bearing mice experienced decreased splenic myelopoiesis.
A, Tumor progression comparing C57BL/6 mice bearing B16F10 tumors with or without NK cell depletion.
B, Differences in CMP/CLP ratio within the spleen of B16F10-bearing mice with or without NK cell depletion.
C and D, Percentage of Ly6C^{high} and Ly6G+ myeloid cells over total CD45+ cells within the spleen of B16F10-bearing mice with or without NK cell depletion.
E, Tumor progression comparing C57BL/6 mice bearing EO771 tumors with or without NK cell depletion.
F, Differences in CMP/CLP ratio within the spleen of EO771-bearing mice with or without NK cell depletion.
G and H, Percentage of Ly6C^{high} and Ly6G+ myeloid cells over total CD45+ cells within the spleen of EO771-bearing mice with or without NK cell depletion.
A to H, NK cells were depleted using anti-NK1.1 depletion antibody in C57BL/6 mice with **(A to D)** B16F10 experiment were conducted with n=6 per group while **(E to H)** EO771 experiment was conducted with n=4 per group.
A and E, One-way ANOVA with multiple comparisons at individual timepoints was used to test for significance. ns= non-significant.
Student t-test was used to test for significance comparing untreated over NK cell-depleted mice in **(B to D)** B16F10-bearing mice and **(F to H)** EO771-bearing mice. *p<0.05, **p<0.01 and ****p<0.0001.



Supplementary Figure 3. Tumor progression in wild type (WT) syngeneic cell line with MHCI-deficient (B2M-KO) cells. **(A)** B16F10 cells was injected into C57BL/6 mice (n=6 per group) while **(B)** 4T1 cells were injected in BALB/c mice (n=7 per group) respectively. One-way ANOVA with multiple comparisons at individual timepoints was used to test for significance. ns= non-significant, *p<0.05, **p<0.01 and ***p<0.001.



Supplementary Figure 4. CRISPR validation of 6 different sgRNA constructs for *B2M*-KO performed in 4T1 cell line. Transduced cells were negatively selected, purified and validated by flow cytometry.



Supplementary Figure 5. Gating strategy for hematopoietic precursors for both bone marrow and spleen FACS analysis.