SUPPLEMENTARY APPENDIX

Inducible deletion of CDK4 and CDK6 – deciphering CDK4/6 inhibitor effects in the hematopoietic system

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Supplementary Information

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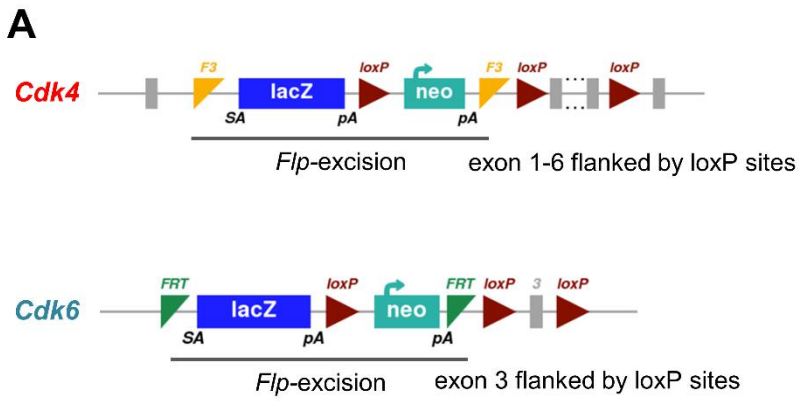
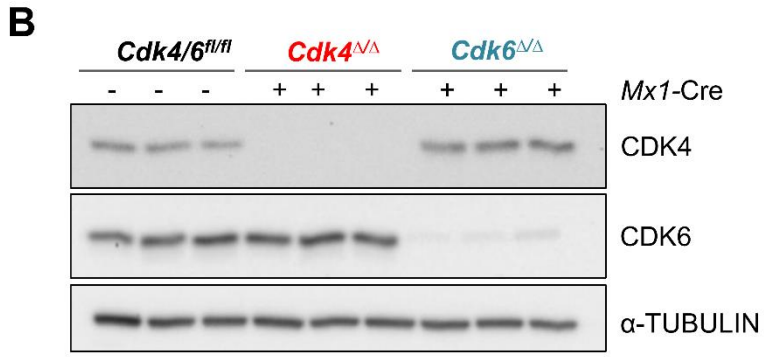
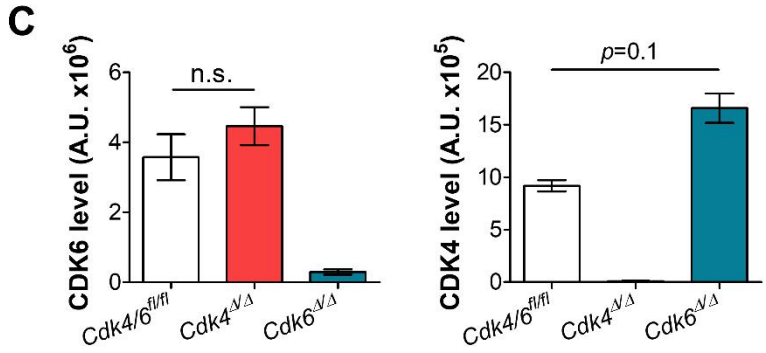
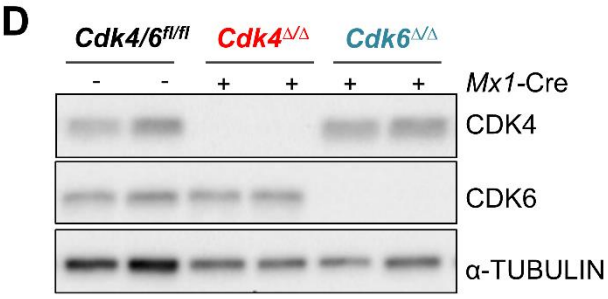




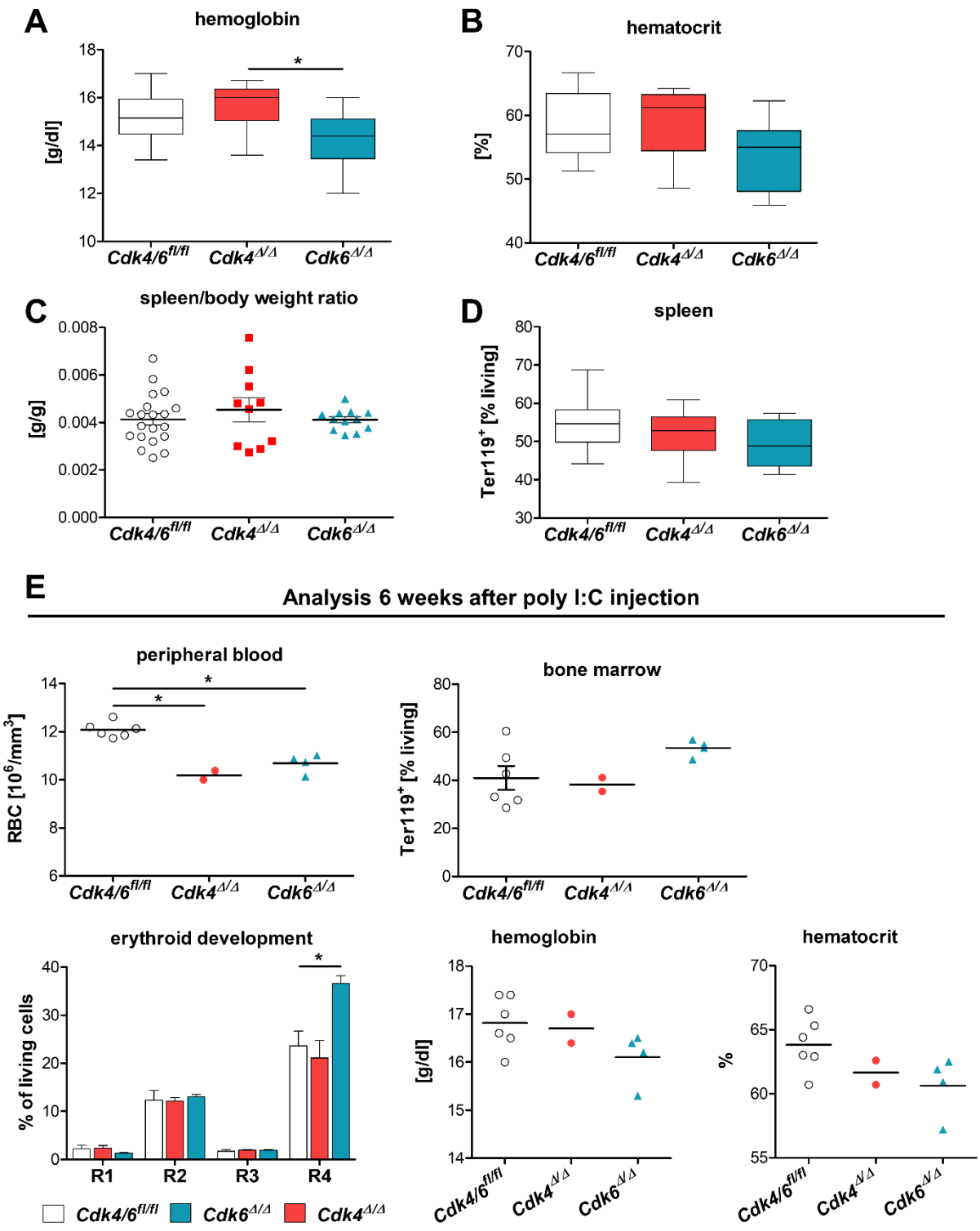




Supplementary Figure 1: Reduced CDK4 or CDK6 expression in *Cdk4*$^{fl/fl}$ or *Cdk6*$^{fl/fl}$ Mx1-Cre mice

(A) Targeting strategy of *Cdk4* (top) or *Cdk6* (bottom). Respective floxed exon(s) were derived by Flp-excision of the neo selection cassette and lacZ reporter. **(B)** Immunoblotting: levels of CDK4 and CDK6 in bone marrow (BM) cells (*Cdk4*$^{fl/fl}$ or *Cdk6*$^{fl/fl}$, *Cdk4*$^{\Delta/\Delta}$, *Cdk6*$^{\Delta/\Delta}$, n=3/genotype). α-TUBULIN served as a loading control. Analysis was performed three weeks post final poly(I:C) injection. A representative blot of at least three independent experiments is shown. **(C)** Quantification of immunoblot shown in (B),

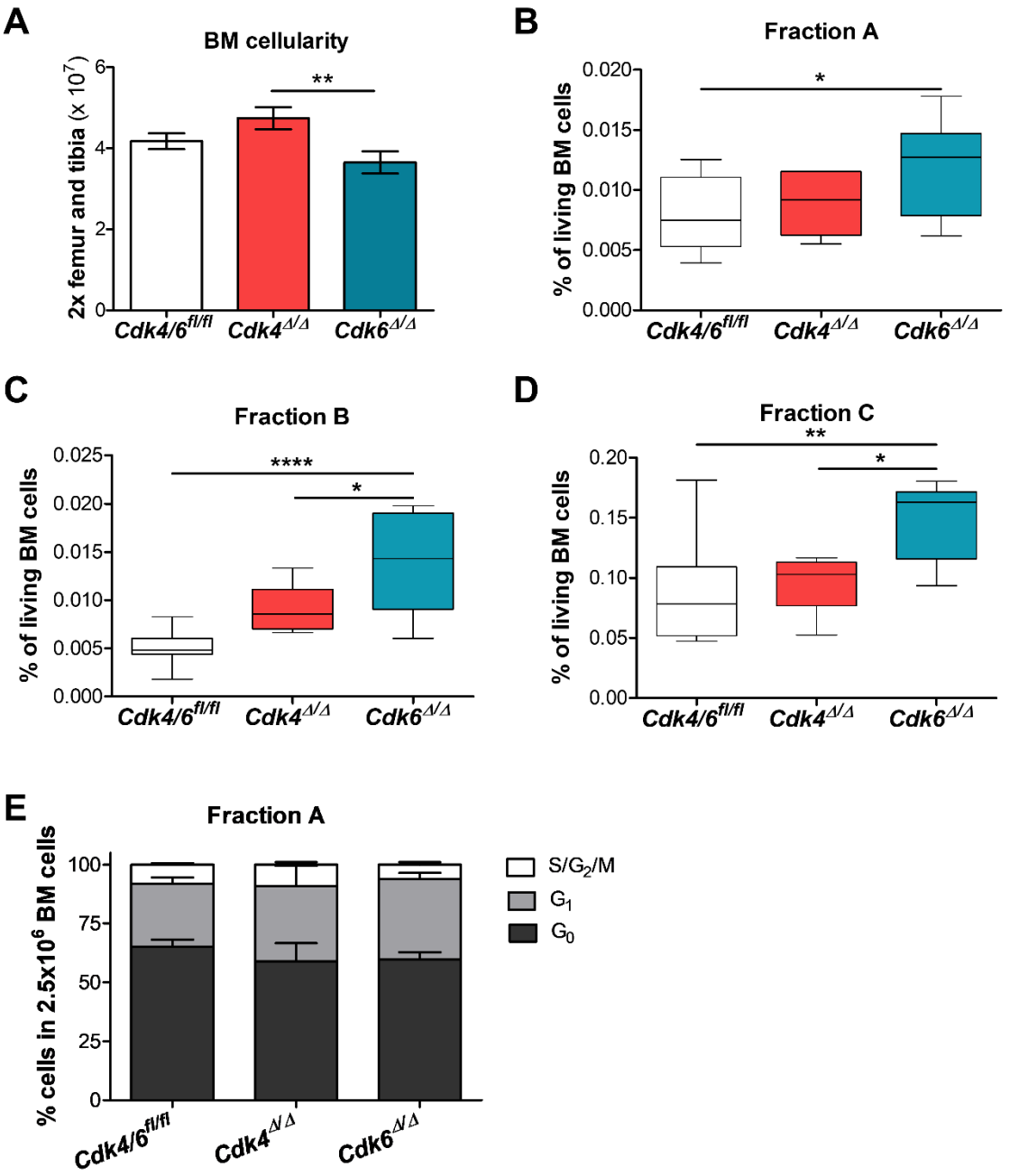
signal intensities were normalized to α-TUBULIN levels (A.U.; arbitrary units). *Cdk4*$^{\Delta/\Delta}$ or *Cdk6*$^{\Delta/\Delta}$ protein levels were compared to the respective controls performing Mann-Whitney U tests. **(D)** Immunoblotting: levels of CDK4 and CDK6 in BM cells (*Cdk4*$^{fl/fl}$ or *Cdk6*$^{fl/fl}$, *Cdk4*$^{\Delta/\Delta}$, *Cdk6*$^{\Delta/\Delta}$, n=2/genotype). α-TUBULIN served as a loading control. Analysis was performed six weeks post final poly(I:C) injection.

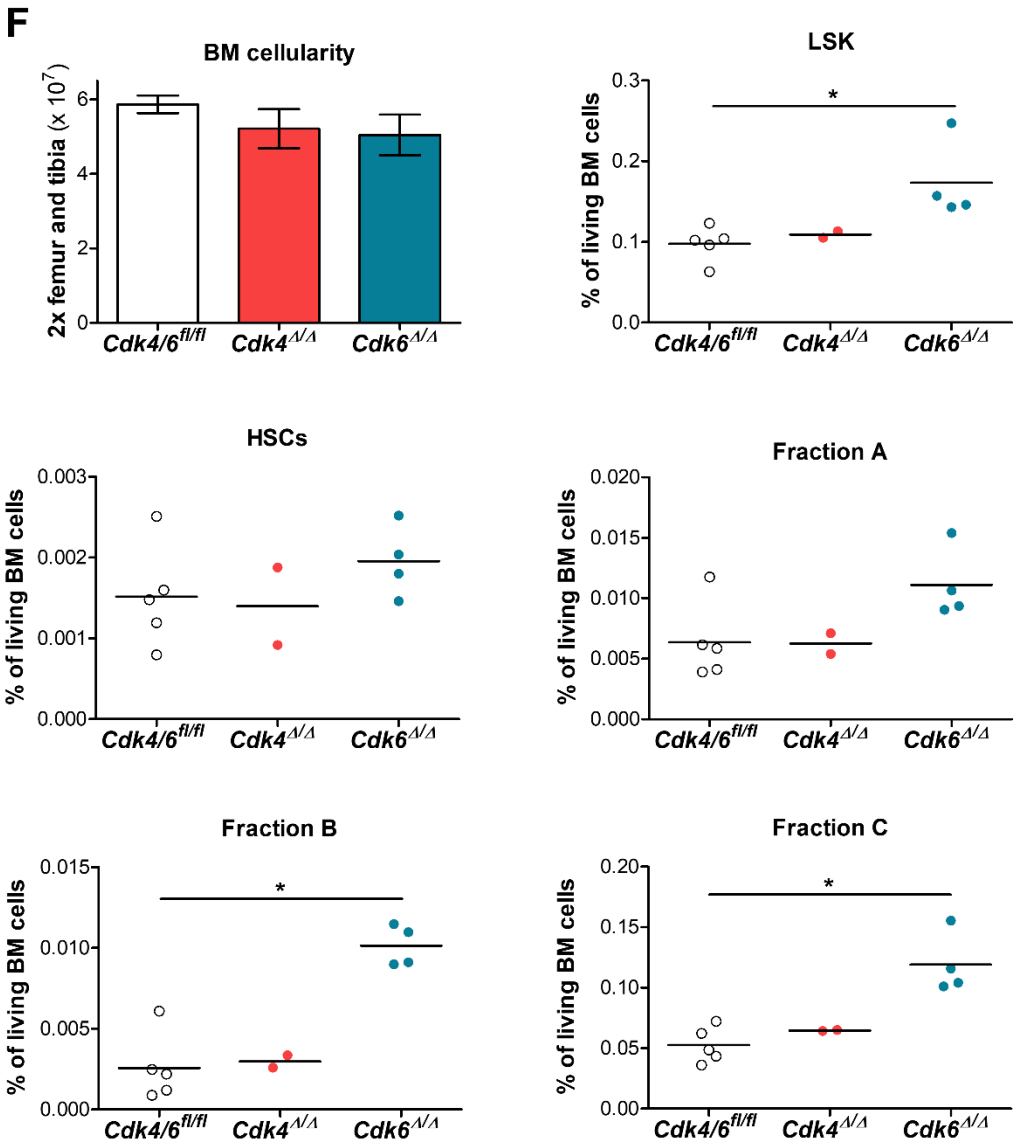


Supplementary Figure 2: *Cdk6*$^{\Delta/\Delta}$ mice are anemic with compensatory upregulation of erythrocytes in the bone marrow

(A-D) Analysis was performed three weeks post final poly(I:C) injection. **(A)** Hemoglobin and **(B)** hematocrit levels in peripheral blood (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥8/genotype). **(C)** Spleen to body weight ratio (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥10/genotype). **(D)** Percentage of Ter119^{+} cells in spleen (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥8/genotype). All comparisons were done with one-way ANOVA followed by Tukey's Multiple Comparison Test, *P≤0.05. (**E**) All analysis shown in Figure 2 and Supplementary

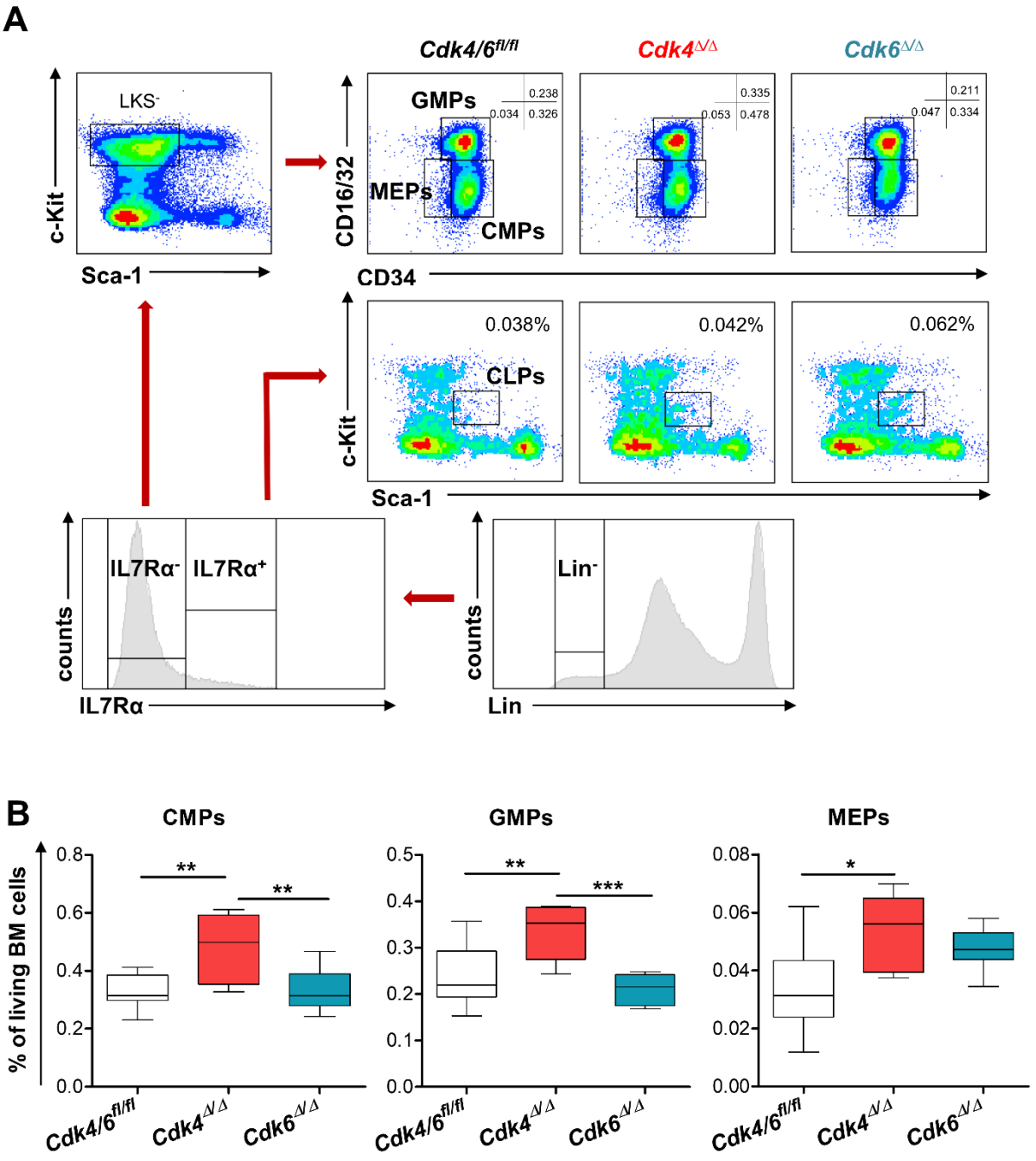
Figure 2A-B were repeated 6 weeks post final poly(I:C) injection. Comparisons were done with Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $*P \leq 0.05$.

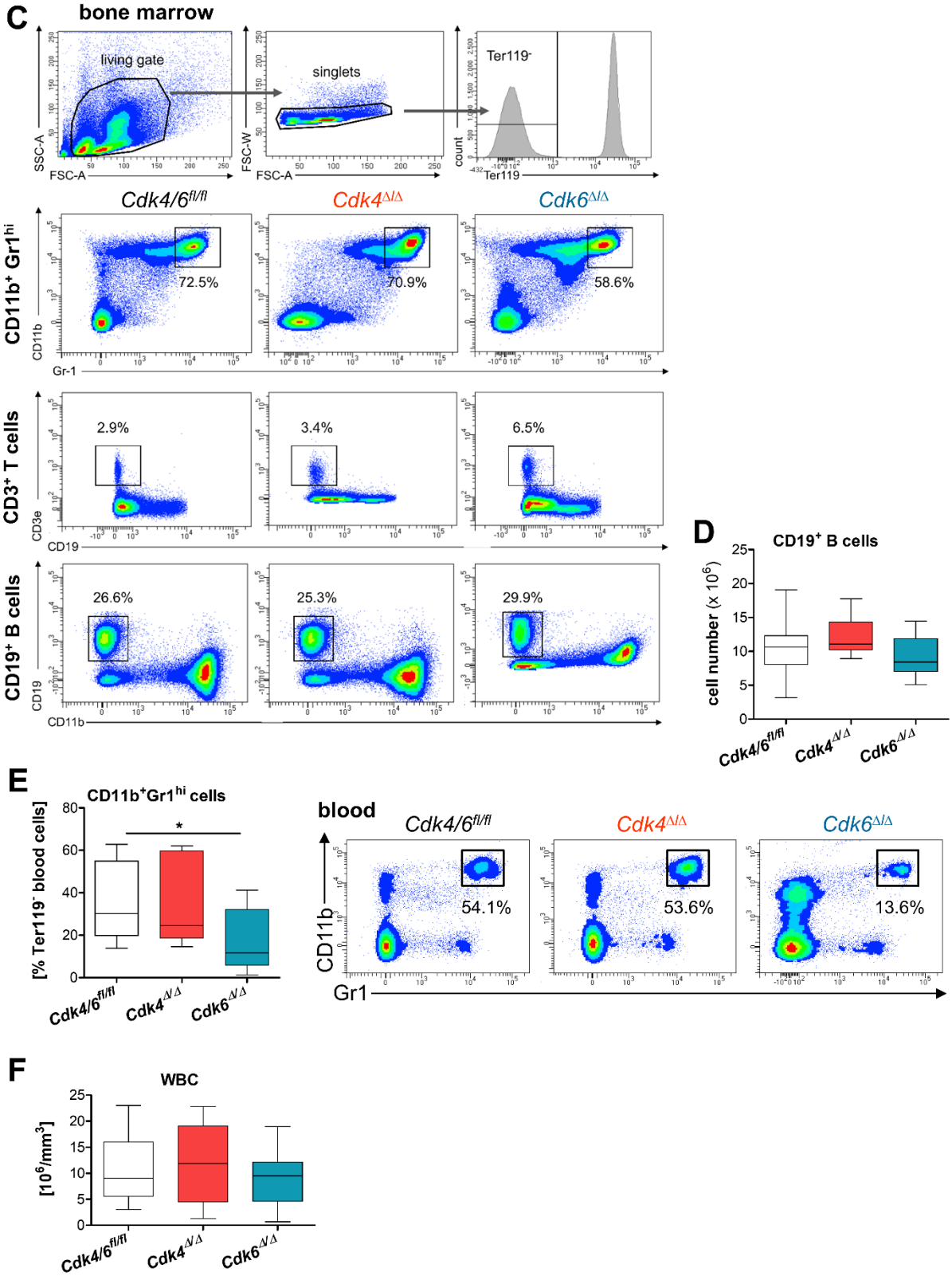

A
BM cellularity
2x femur and tibia (x 10^7)
**
Cdk4/6fl/fl
Cdk4Δ/Δ
Cdk6Δ/Δ
B
Fraction A
% of living BM cells
*
C
Fraction B
****
*
D
Fraction C
**
*
E
Fraction A
% cells in 2.5x10^6 BM cells
S/G2/M
G1
G0

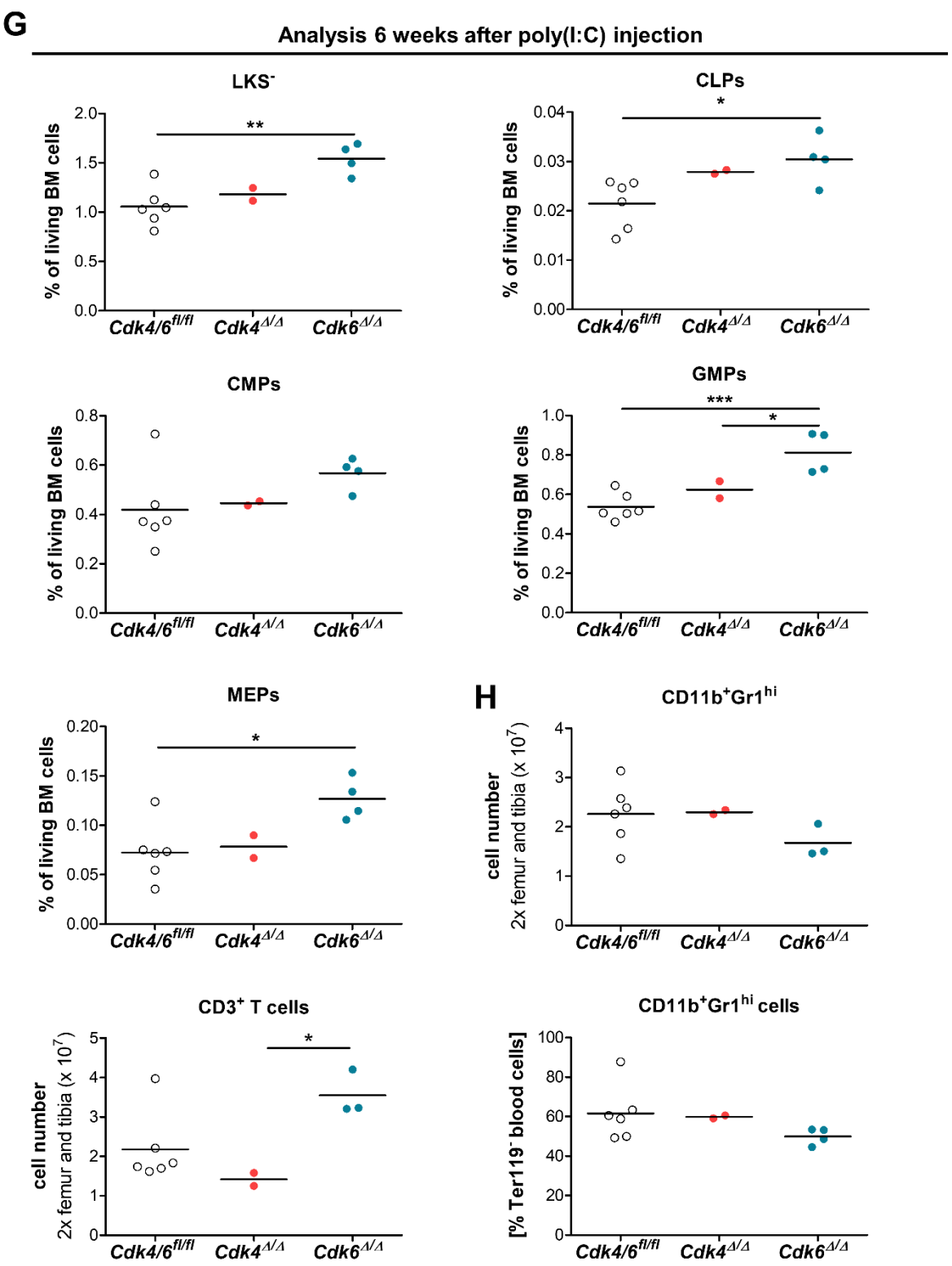


Supplementary Figure 3: CDK6 alters the composition of the hematopoietic stem and progenitor pool

(A-E) Analysis was performed three weeks post final poly(I:C) injection. **(A)** Bone marrow (BM) cellularity in both femur and tibia per mouse (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥6/genotype). Percentage of **(B)** Fraction A (CD150^{+}CD48^{-}LSKs), **(C)** Fraction B (CD150^{+}CD48^{+}LSKs) and **(D)** Fraction C (CD150^{-}CD48^{+}LSKs) hematopoietic stem cells per 2.5*10^{6} BM cells (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥5/genotype). **(E)** DAPI/Ki-67 cell cycle staining of Fraction A cells. Depicted are % cells G_0, G_1 or $S/G_2/M$ per 2.5*10^{6} BM cells (n=3 (*Cdk4*$^{\Delta/\Delta}$); n=3 (*Cdk6*$^{\Delta/\Delta}$); n=6 (*Cdk4/6*$^{fl/fl}$)). All comparisons were done with one-way ANOVA followed by Bonferroni´s Multiple Comparison Test, *P≤0.05, **P≤0.01, ***P≤0.001. **(F)** Analysis six weeks post final poly(I:C) injection: BM cellularity in both femur and tibia per mouse. Percentage of LSKs, Fraction A, Fraction B and Fraction C cells and hematopoietic stem cells (HSCs) (CD150^{+}CD48^{-}CD135^{-}CD34^{-}LSKs) per 2.5*10^{6} BM cells. Comparisons were done with Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, *P≤0.05.


A
Cdk4/6fl/fl
Cdk4Δ/Δ
Cdk6Δ/Δ
LKS-
c-Kit
Sca-1
CD16/32
CD34
GMPs
MEPs
CMPs
0.238
0.034
0.326
0.335
0.053
0.478
0.211
0.047
0.334
0.038%
0.042%
0.062%
CLPs
IL7Rα-
IL7Rα+
Lin-
counts
IL7Rα
Lin
B
CMPs
GMPs
MEPs
% of living BM cells
0.8
0.6
0.4
0.2
0.0
0.5
0.3
0.1
0.08
0.06
0.04
0.02
0.00
**
***
*
Cdk4/6fl/fl
Cdk4Δ/Δ
Cdk6Δ/Δ


C
bone marrow
living gate
singlets
Ter119-
SSC-A
FSC-A
FSC-W
count
Ter119
Cdk4/6fl/fl
Cdk4Δ/Δ
Cdk6Δ/Δ
CD11b+ Gr1hi
72.5%
70.9%
58.6%
CD11b
Gr-1
CD3+ T cells
2.9%
3.4%
6.5%
CD3e
CD19
CD19+ B cells
26.6%
25.3%
29.9%
CD19
CD11b
D
CD19+ B cells
cell number (x 10^6)
E
CD11b+Gr1hi cells
[% Ter119- blood cells]
*
blood
54.1%
53.6%
13.6%
F
WBC
[10^6/mm^3]



Supplementary Figure 4: CDK4 and CDK6 drive the myeloid and lymphoid progenitor pool in opposite directions

(A-F) Analysis was performed three weeks post final poly(I:C) injection. **(A)** Representative flow cytometry gating strategy of common lymphoid progenitors (CLPs), myeloid-primed progenitors (Lin-c-Kit+Sca-1; LKS-), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs) in *Cdk4/6*$^{fl/fl}$, *Cdk4*$^{\Delta/\Delta}$ and *Cdk6*$^{\Delta/\Delta}$ bone marrow (BM). **(B)** Percentage of CMPs (LKS$^-$CD34$^+$CD16/32^{lo}), GMPs (LKS$^-$CD34$^+$CD16/32^{hi}) and MEPs (LKS$^-$CD34$^-$CD16/32^{lo}) per 1*10^6 BM cells (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥5/genotype). **(C)** Representative flow

cytometry gating strategy of $CD11b^{+}Gr1^{hi}$ cells, $CD3^{+}$ T cells and $CD19^{+}$ B cells in *Cdk4/6*$^{fl/fl}$, *Cdk4*$^{\Delta/\Delta}$ and *Cdk6*$^{\Delta/\Delta}$ BM. **(D)** $CD19^{+}$ B cells in BM (2 femurs and tibias; *Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥8/genotype). **(E)** Left: Relative numbers of $CD11b^{+}Gr1^{+}$ myeloid cells in blood (gated on $Ter119^{-}$ cells; *Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥8/genotype). Right: Representative flow cytometry gating. **(F)** White blood cells (WBC) in peripheral blood (*Cdk4*$^{\Delta/\Delta}$; *Cdk6*$^{\Delta/\Delta}$; *Cdk4/6*$^{fl/fl}$, n≥15/genotype). All comparisons were done with one-way ANOVA followed by Bonferroni´s Multiple Comparison Test, **P*≤0.05, ***P*≤0.01, ****P*≤0.001. Analyses shown in **(G)** Figure 4A-B, Supplementary Figure 4A-B, **(H)** Figure 4C-D and Supplementary Figure 4E were repeated six weeks after the final poly(I:C) injection. Comparisons were done with Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, **P*≤0.05; ** *P*≤0.01.

Supplementary Methods

Genotyping and deletion PCR

***Cdk4*$^{fl/fl}$**: Genomic DNA was amplified with the following primers: *Cdk4*_wt (forward: TGCAGAATCTTCGGTGCAAACCCTG, reverse: ATGACCCGGCGGTAACAAAGGAACTC) resulting in a 244 bp product in mice harboring a wildtype allele, and *Cdk4*_loxP (forward: GCGCAACGCAATTAATGATAAC, reverse: GCAGTGACAACTACAGCCTGCCCAC) resulting in a 346 bp product in mice harboring a loxP-flanked *Cdk4* allele at the following conditions: 3 min 95 °C; 35 x 95 °C 15 sec., 60 °C 15 sec., 72 °C 20sec; 72 °C 1 min, hold at 8 °C. Using the *Cdk4*_wt forward and *Cdk4*_loxP reverse primer, the deletion band (*Cdk4*$^{\Delta/\Delta}$, ~ 400 bp) can be amplified.

***Cdk6*$^{fl/fl}$:** Genomic DNA was amplified with the following primers: *Cdk6*_tm1c_wt (forward: *ATCCATGTTTGGAGCACCTTTGGAGAG*, reverse: *TGAGCCAACGTACTGCCCTAGCAATG*) resulting in a 245 bp product in mice harboring a wildtype allele and a 430 bp product in mice harboring a loxP-flanked *Cdk6* allele at the following conditions: 3 min 95 °C; 35 x 95 °C 15 sec., 60 °C 15 sec., 72 °C 20sec; 72 °C 1 min, hold at 8 °C. By adding the *Cdk6*_delta primer (*AACAGAAAGGGCCAGATGACCATGC*), the deletion band (*Cdk6*$^{\Delta/\Delta}$, ~ 600 bp) can be amplified.

Hematocytometry

Peripheral blood was collected by *vena facialis* puncture into EDTA-coated tubes (Mini-Collect K3EDTA tubes, Greiner Bio-One). Automated complete blood counts were obtained from a hematology analyzer (scil Vet ABC). Composition of immune cells was analyzed by flow cytometry.

Immunoblotting

Whole-cell lysates of BM or spleen were lysed in 1x Laemmli buffer, incubated at 95°C for 5 min and sonicated for 15 min at RT. Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Proteins were resolved by a 10% sodium dodecyl sulphate polyacrylamide gel and transferred to Immobilon®-P Polyvinylidene difluoride membrane (Merck, Darmstadt, Germany). Membranes were blocked in 5% milk for 1 h and probed with the appropriate antibody overnight. Detection of bound antibodies was performed by incubation with horseradish peroxidase-conjugated anti-rabbit (Cell Signaling Technology, Danvers, MA, USA, 7074S) or anti-mouse (CST, 7076S) antibodies at room temperature for 1 h followed by chemiluminescent visualization

after incubation of the membranes with 20X LumiGLO® Reagent and 20X Peroxide (CST) using the ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA, USA) according to manufacturer´s protocol. Anti-CDK4 (C-22), anti-CDK6 (SPM 383) and α-TUBULIN (DM1A) were obtained from Santa Cruz (Dallas, Texas, USA). Immunoblots were quantified using Image Lab 5.2.1 software (Bio-Rad).