Supplementary Information

CD4+ T cells, but not non-classical monocytes, are dispensable for the development of chronic lymphocytic leukemia in the TCL1-tg murine model.

Supplementary Methods

Animal ethics

Animal experiments were performed under approval from the central Austrian animal ethics committee. The licenses for animal experimentation, BMWF 66.012/0009-II/3b/2012, 20901-TGV/52/11-2012, and 20901-TVG/102/6-2015 are held by Dr. Alexander Egle.

Mouse strains

All transgenic mouse strains were maintained by crossing to C57BL/6 mice. The original TCL1-tg mice [Bichi *et al*, 2005] were obtained from C. Croce (Columbus, Ohio, USA) and were backcrossed to genetic homogeneity (>10 generations) in the C57BL/6 background in order to make it amenable to genetic manipulation without complications in data analyses arising from genetic variations between strains. Furthermore, this has allowed us to successfully establish an adoptive transfer model transplanting tumor cells from leukemic mice into congenic recipients without the need for immunosuppression of the recipients [Hofbauer *et al*, 2011]. The development of CLL in these mice is heterogenous and mice additionally develop secondary malignancies [Zanesi *et al*, 2006]

GK5 transgenic mice (C57BL/6 background, hereafter referred to as GK mice) were a kind gift from Andrew M. Lew of the Walter and Eliza Hall Institute (Melbourne, Australia). GK mice carry a transgene that encodes an anti-CD4 antibody (Ab) under the control of the cytomegalovirus (CMV) promoter, leading to the continuous secretion of anti-CD4 antibodies mainly by the exocrine pancreas and muscle [Han *et al*, 2000; Zhan *et al*, 2000], and results in the complete and permanent clearance of CD4-expressing cells in the periphery [Han *et al*, 2000].

Other *in vivo* models of CD4+ T cell-deficiency include exogenously injecting mice with anti-CD4 to Ab deplete these cells, and mice bearing targeted deletions of either the CD4 gene (CD4-/-) [Rahemtulla *et al*, 1991; Locksley *et al*, 1993] or the major histocompatibility complex (MHC) class II gene (class II-/-) [Cardell *et al*, 1994; Grusby *et al*, 1995]. However, we chose the GK model for several reasons. First, CD4 depletion by injection of exogenously supplied anti-CD4

antibody is both logistically and financially unfeasible for long-term experiments. Furthermore it has been demonstrated that the efficiency of CD4-depletion via this strategy can vary according to the activation status of CD4+ T cells. Activated and memory CD4+ T cells, which accumulate in aged mice, were shown to be resistant to *in vivo* depletion by anti-CD4 Ab [Chace *et al*, 1994; Rice *et al*, 1995]. Second, both CD4-/- and class II-/- mice demonstrate defects in T cell development in the thymus, as both CD4 and Class II MHC molecules are necessary for the proper maturation of CD4+ T cells. These defects are compensated for by the development of unconventional T cell subsets. For example, in CD4-/- mice, increased numbers of CD4-CD8-TCR$\alpha\beta$+ T cells (DN) are observed which appear to function as bona fide helper cells, are MHC II – restricted, and are functionally different from cells bearing the same immunophenotype in wild type (WT) mice. In class II -/- mice, a significant residual CD4+ population remains that is CD1-restricted, and the CD8+ population is significantly enhanced. Finally, while B cell development and differentiation is normal in CD4-/- mice, T-dependent antigen driven germinal center formation could be observed which is likely mediated by the DN MHC II-restricted population. In class II-/- mice, germinal center formation was absent, however considerably lower numbers of B cells were observed.

In contrast to these CD4-deficient mouse models, GK mice exhibit no defects in thymic T cell maturation. The cytometric profile of the thymus in GK mice is similar to WT mice [Zhan *et al*, 2003]. Instead, as mature CD4+ T cells exit the thymus, they are completely and permanently depleted from the periphery by the continuous endogenous production of anti-CD4 Ab. None of the unconventional T cell subsets observed in other models of CD4-deficiency were seen in GK mice. Additionally, activated and or memory T cells, which are resistant to antibody-depletion, never develop in the GK mice. Data regarding the population of NK T cells showed that this population is normal in size and that the DN subset of NK T cells seem to compensate for the loss of CD4+ NK T cells [Zhan *et al*, 2004]. A subset of dentritic cells (DCs) and macrophages also express CD4 [Shortman *et al*, 2002] and are therefore absent in GK mice, which is reflected by reduced total numbers of DCs. Finally, B cell development is normal in these mice, with no reduction in absolute B cell numbers observed. However, germinal center formation is absent due to the loss of peripheral CD4+ T cells which are critical for germinal center formation, Ig gene somatic hypermutation, and B cell memory development [Zhan *et al*, 2004]. In this context, GK mice have been used together with the VavP-Bcl2-tg murine model for follicular lymphoma to demonstrate that the dependence of germinal center expansion observed in these mice is dependent on CD4+ T cell help [Egle *et al*, 2004]. Thus, of all the CD4-deficient mouse models available, and given the precedent of using these mice in the context of a murine lymphoma

model, GK mice represented the most interesting tool to use in conjunction with TCL1 transgenic mice to investigate the role of CD4+ T cell dependent germinal center-like reactions in the establishment of a malignant CLL clone.

Adoptive transfer and primary disease models

For adoptive transfer experiments, between 10 – 20 x 10^6 white blood cells derived from spleens of leukemic mice were injected intraperitoneally into 6 – 8 week old recipient mice unless otherwise noted. GK and their WT littermate controls were used as recipients in adoptive transfer experiments.

For the primary disease model TCL1-tg mice were crossed to GK mice thereby generating GK/TCL1-tg, GK, TCL1-tg, and WT mice. Mice of all resulting genotypes were followed for development of CLL in overall survival analyses.

In both disease models development of CLL was monitored in the peripheral blood at regular intervals and animals were sacrificed at designated humane endpoints. Development of CLL was confirmed by the presence of >5000 CLL cells per µl blood. Upon sacrifice, peripheral blood, peritoneal cavity wash, spleen, lymph nodes, bone marrow, and liver were harvested and single cell suspensions prepared for further analyses.

Flow cytometry

All antibodies used in this study were purchased from BD Biosciences, eBioscience, or Biolegend and are listed below. To analyse the organs of sick mice single cell suspensions of various lymphoid organs were stained with antibodies towards CD5 (FITC), CD25 (PE), CD44 (PerCP-Cy5.5), CD62L (PC7), CD4 (APC), CD19 (AlexaFluor700), and CD8 (APC-H7). The relative percentage of CLL cells in each organ was identified based on their expression of CD5 and CD19.

To measure tumor development in the blood, we used a mastermix of antibodies against CD3 (FITC), CD19 (PE), CD5 (PC5), CD8a (PC7) and CD4 (PB or eFluor450), or a mastermix containing CD4 (FITC), CD19 (PE), CD5 (PC5) and CD8a (PC7) –targeting antibodies. To analyse monocyte subsets and tumor development simultaneously in the blood we used a mastermix of CD5 (PC7), CD19 (PB or eFluor450), CD14 or CD115 (APC), CD43 (FITC) and Gr1 (PE) – targeting antibodies. Briefly, blood was drawn from the tail vein and collected into tubes containing 0.02M EDTA. Peripheral blood samples were incubated with the antibody

mastermixes described above and 15 – 20 µl of Flow-Count Fluorospheres (Beckman Coulter) in a total staining volume of 100 µl. Cells were stained for 15 min in the dark. After staining, erythrocytes were lysed by the addition of 2 ml FACS lysis buffer and brief vortexing at 1400 rpm. Cells were pelleted by centrifugation for 5 min at 350 x g. The supernatant was discarded and cells were resuspended in 200 µl PBS for analysis on a Beckman Coulter Galios. Absolute cell numbers were calculated with reference to the number of count beads acquired.

Antibody	Clone	Conjugate	Catalog #	Company
CD3	145-2C11	FITC	100306	Biolegend
CD4	RM4-5	eFluor450	48-0042	eBiosciences
CD4	RM4-5	APC	100516	Biolegend
CD4	H129.19	FITC	553651	BD Biosciences
CD5	53-7.3	PE/Cy5	100610	Biolegend
CD5	53-7.3	FITC	11-0051	eBiosciences
CD5	53-7.3	PE/Cy7	25-0051	eBiosciences
CD8a	53-6.7	PE/Cy7	552877	BD Biosciences
CD8a	53-6.7	APC-H7	560182	BD Biosciences
CD8a	53-6.7	PB	100725	Biolegend
CD8a	53-6.7	PE/Cy7	100722	Biolegend
CD14	Sa-2-8	APC	17-0141	eBiosciences
CD19	eBio1D3	Alexa Fluor 700	56-0193	eBiosciences
CD19	eBio1D3	eFluor 450	48-0193	eBiosciences
CD19	6D5	PB	115523	Biolegend
CD19	6D5	PE	115508	Biolegend
CD25	PC61	PE	102008	Biolegend
CD25	PC61.5	PE	12-0251	eBiosciences
CD43	S7	FITC	553270	BD Biosciences
CD44	IM7	PerCP/Cy5.5	103032	Biolegend
CD62L	MEL-14	PE/Cy7	104418	Biolegend
CD115	AFS98	APC	135510	Biolegend
Gr1	RB6-8C5	PE	108408	Biolegend

BCR IgVH CDR3 Spectratyping.

CD5+ populations were isolated from splenocyte cell suspensions prepared from TCL1-tg and GK/TCL1-tg mice by magnetic bead sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). As a control CD19+ cells were isolated from splenocyte cell suspension from WT mice. RNA was extracted from sorted cells using the Qiagen Blood RNA kit. In all, 0.5–1.0 µg of total RNA was used to generate cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria). cDNA samples were amplified in a touchdown PCR using a degenerate primer set (MH1-7, below) to amplify all mouse VH genes of the BCR repertoire [Wang *et al*, 2000], and a degenerate AF 647 or FAM labeled primer (JHR, below) for JH segments 1 to 4. The primers were as follows: MH1, 5′-SARGTNMAGCTGSAGSAGTC-3′; MH-2, 5′-SARGTNMAGCTGSAGSAGTCWGG-3′; MH-3, 5′-CAGGTTACTCTGAAAGWGTSTG-3′; MH-4, 5′-GAGGTCCARCTGCA ACARTC-3′; MH-5, 5′-CAGGTCCAACTVCAGCARCC-3′; MH-6, 5′-GAGGTGA ASSTGGTGGAATC-3′; MH-7, 5′-GATGTGAACTTGGAAGTGTC-3′; JHR, 5′-CCTGMRGAGACDGTGASHRDRGTBCCTKKRCCCC-3′ (using the IUB mixed base nomenclature in the degenerate primers). The following PCR conditions were used.

Cycles	Temperature	Time
1x	95°C	15 min
16x (touchdown)	95°C	45 sec
	65°C (-0.5°C/cycle)	45 sec
	72°C	50 sec
30x	94°C	45 sec
	57°C	45 sec
	72°C	50 sec
1x	72°C	10 min

For fragment analyses each PCR reaction was diluted 1:500 in H_2O and prepared for capillary-electrophoresis as follows: 12 µl Hi-Di Formamide, 1 µl DNA sample and 0,5 µl DNA Size Standard 550 (BTO) was added to PCR tubes. The sample was denatured for at least 3 min at 95°C and analysed on the ABI Genetic Analyzer 3500 (Applied Biosystems). The clonality of each BCR BV family was determined semi-quantitatively from the distribution of the CDR3 lengths (visualized as peaks) obtained.

In vivo monocyte/macrophage depletion study

10-week old GK mice (*n* = 10) and their WT littermate controls (*n* = 10) were equally assigned into one of 4 cohorts: GK/Clodrosome® (*n* = 5), GK/Encapsome® (*n* = 5), WT/Clodrosome® (*n* = 5), and WT/Encapsome® (*n* = 5). No specific method for randomization was used to allocate the mice into the different treatment cohorts. Our only criteria were: equivalent distribution of genotypes in each treatment arm, equivalent distribution of gender in each treatment arm, and that each cage should house mice in both treatment arms. Liposomal clodronate (Clodrosome®) and control liposomes (Encapsome®) were purchased from Encapsula NanoSciences LLC (Brentwood, TN, USA) and administered to mice according to manufacturer's protocols. Briefly, 1 mg Clodronate disodium salt (200 µl Clodrosome®), or an equivalent volume of control liposomes (Encapsome®), was injected intraperitoneally into mice twice a week (Monday and Thursday). Mice were inoculated with tumor cells (approx. 7 x 10^6 white blood cells) one day after the first Clodrosome® / Encapsome® injection. Monocyte subsets and tumor development were monitored by flow cytometric analyses of blood samples drawn once weekly. In the first week, blood was drawn prior to administration of Clodrosome® or Encapsome® (Monday) to obtain a baseline measurement of the different immune subsets. In all subsequent weeks, blood was drawn on a Friday, so that in week 2 blood was drawn one day after Clodrosome® or Encapsome® injection. We observed that Clodrosome® (but not Encapsome®) treatment resulted in the thickening of the skin around the injection site, making it difficult to perform continued intraperitoneal injections and administer the correct dose after 2 weeks (originally, 3 weeks of treatment had been planned). For these reasons, treatment was discontinued and tumor development was followed without further treatment of the mice. The study was terminated on week 8 when all mice were sacrificed within a one-week period.

Blinding

Investigators were not blinded to the mouse genotype or treatment allocation in any of the animal experiments.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5.0 Software. Data are represented either as box-and-whiskers graphs, where the box extends from the first to third quartiles, the band within the box represents the median, and the whiskers represent the minimum and maximum values, or as stacked columns wherein the median and range are plotted. Where relevant, data were analysed for normal distribution using the Kolmogorov-

Smirnov test. Where the data in the two groups to be compared were normaly distributed, the unpaired t-test (two-tailed) was used. Where the data to be compared were not normaly distributed, the Mann-Whitney test (two-tailed) was used. Results were considered significant when $P < 0.05$. Significance levels are denoted by asterisks in graphs with $P < 0.05$ marked as *, <0.01 as **, and < 0.001 as ***; "n.s." = not significant.

For survival analyses (primary disease and adoptive transfer models), we estimated needing cohort sizes of 10 - 14 mice using the algorithm http://www.stat.ubc.ca/~rollin/stats/ssize/n1.html. Overall survival curves were plotted by Kaplan-Meier and the survival distributions of the different cohorts were compared using log-rank (Mantel-Cox) test. Death was scored as a CLL event when the absolute number of CLL cells measured >5000 per µl blood at the end stage of disease, or when the CLL fraction was prominent in at least 3 out of the 5 lymphoid compartments analysed. For subsequent analyses of splenomegaly, extent of tumor cell infiltration into the different lymphoid organs in TCL1-tg and GK/TCL1-tg mice, and BCR spectratyping of tumors, only mice whose death was scored as a CLL event were included. For analyses of subset distribution of circulating peripheral blood monocytes, only mice whose death was scored as a CLL event were included in the leukemic cohorts.

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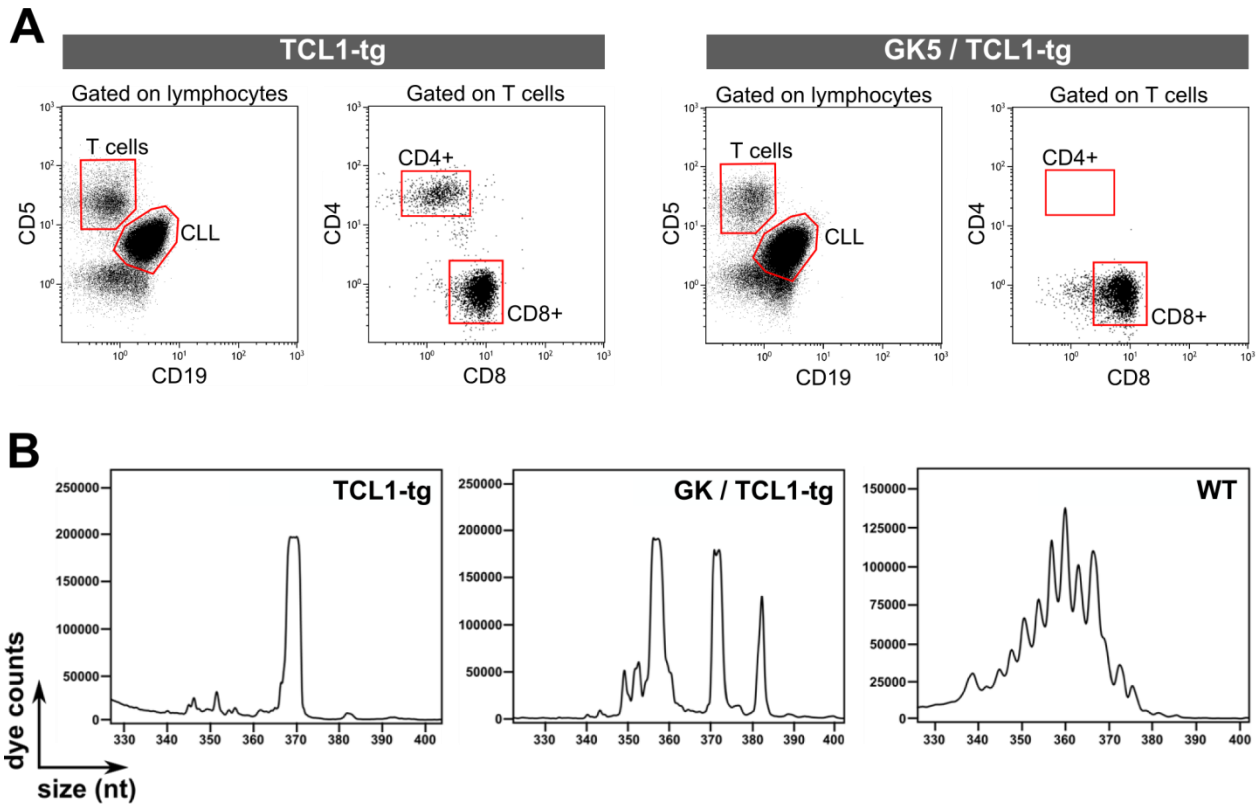
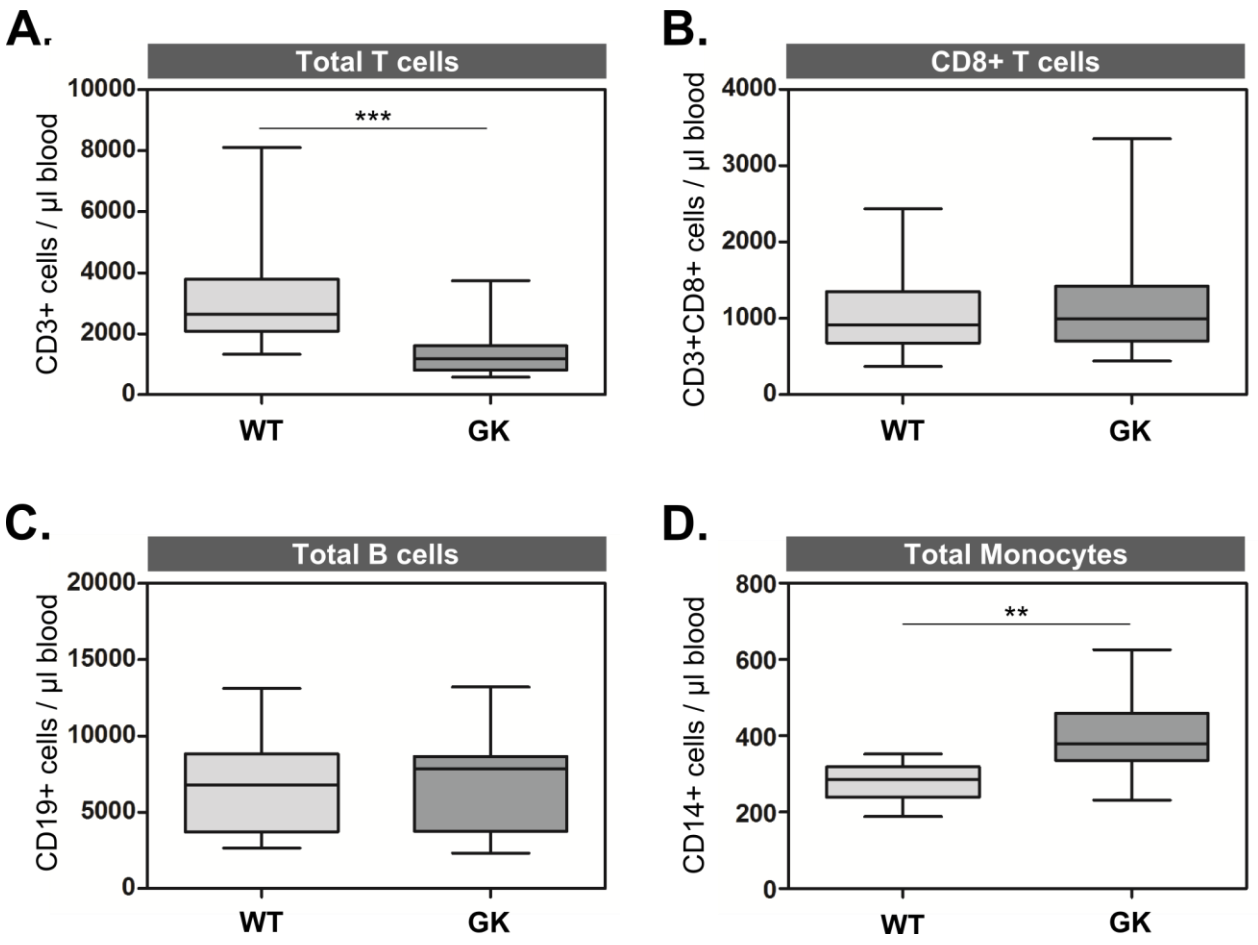
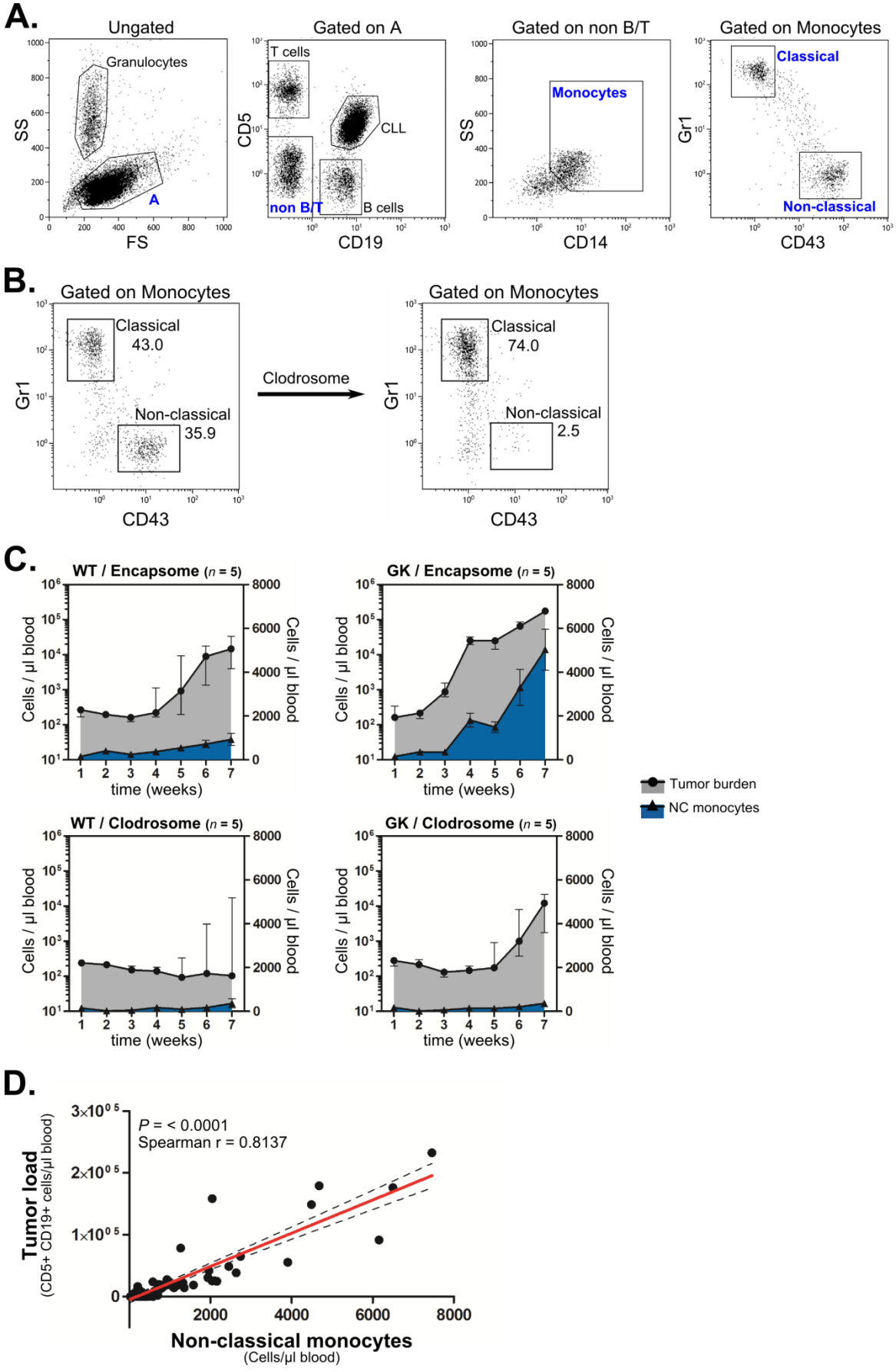


Supplementary Figure 1. CLL develops in TCL1-tg mice in the absence of CD4+ T cells. (A) Gating strategy for flow cytometric analyses of peripheral blood leukocytes in leukemic mice. CLL cells are CD19+ CD5+ whereas T cells are CD19- CD5+. T cells are further separated into CD4+ and CD8+ T cells. CD4+ T cells are absent in GK / TCL1-tg mice (right panel). (B) BCR clonality of CLL tumors arising in TCL1-tg and GK/TCL1-tg mice. CD5+ B cells isolated from spleens of leukemic mice (TCL1-tg or GK/TCL1-tg) display mono- and oligoclonal patterns in CDR3 spectratyping analyses compared to the polyclonal pattern obtained from CD19+ B cells isolated from age-matched WT mice.



Supplementary Figure 2. Monocytes are increased in GK mice. Comprehensive immunophenotyping analyses was performed in peripheral blood samples obtained from WT (*n* = 9) and GK (*n* = 10) mice. Absolute numbers of total T cells are decreased in GK mice (A) due to the loss of CD4+ T cells, whereas absolute CD8+ T cell numbers are similar between the two genotypes (B). Total B cell numbers are similar between WT and GK mice (C), but absolute monocyte numbers are increased in GK mice compared to WT controls (D).


A.
Ungated
Granulocytes
A
SS
FS
Gated on A
T cells
CLL
non B/T
B cells
CD5
CD19
Gated on non B/T
Monocytes
SS
CD14
Gated on Monocytes
Classical
Non-classical
Gr1
CD43
B.
Gated on Monocytes
Classical
43.0
Non-classical
35.9
Gr1
CD43
Clodrosome
Gated on Monocytes
Classical
74.0
Non-classical
2.5
Gr1
CD43
C.
WT / Encapsome (n = 5)
GK / Encapsome (n = 5)
WT / Clodrosome (n = 5)
GK / Clodrosome (n = 5)
Cells / µl blood
time (weeks)
Tumor burden
NC monocytes
D.
P = < 0.0001
Spearman r = 0.8137
Tumor load
(CD5+ CD19+ cells/µl blood)
Non-classical monocytes
(Cells/µl blood)

Supplementary Figure 3. Non-classical monocytes impact CLL development in mice. (A) Gating strategy to distiniguish between classical (Gr1++ CD43+) and non-classical (Gr1+ CD43++) monocytes in peripheral blood based on nomenclature proposed by Zeigler-Heitbrock et al (Ref 11, main text). (B) Treatment with Clodrosome® results in selective depletion of non-classical monocytes. (C) CLL development in mice engrafted with tumor cells is accompanied by an increase in absolute numbers of non-classical monocytes (WT/Encapsome and GK/Encapsome). Treatment of mice with Clodrosome® resulted in reduced absolute numbers of non-classical monocytes and significantly delayed tumor development (WT/Clodrosome and GK/Clodrosome). (D) The absolute number of CD5+ CD19+ cells positively correlates with the absolute number of non-classical monocytes.