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-TCRαβ+ 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 dendritic 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 10⁶ 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′-CAGGTTACTCTGAAGWGTSTG-3′; MH-4, 5′-GAGGTCARCTGCA ACARTC-3′; MH-5, 5′-CAGGTCAACTVCAGCARCC-3′; MH-6, 5′-GAGGTGA ASSTGGTGGAAATC-3′; MH-7, 5′-GATGTGAACTTGGGAAGTGTC-3′; JHR, 5′-CCTGMRAGACDGTSASHRDRTGTCCTTKRCC-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 H₂O 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 10⁶ 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 normally distributed, the unpaired t-test (two-tailed) was used. Where the data to be compared were not normally 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](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).
Supplementary Figure 3. Non-classical monocytes impact CLL development in mice. (A) Gating strategy to distinguish 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.