TBET-expressing Th1 CD4+ T cells accumulate in chronic lymphocytic leukaemia without affecting disease progression in Eμ-TCL1 mice

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Summary
Chronic lymphocytic leukaemia (CLL) is associated with alterations in T cell number, subset distribution and function. Among these changes, an increase in CD4+ T cells was reported. CD4+ T cells are a heterogeneous population and distinct subsets have been described to exert pro- and anti-tumour functions. In CLL, controversial reports describing the dominance of IFNγ-expressing Th1 T cells or of IL-4-producing Th2 T cells exist. Our study shows that blood of CLL patients is enriched in Th1 T cells producing high amounts of IFNγ. Moreover, we observed that their frequency remains relatively stable in CLL patients over a time course of five years. Furthermore, we provide evidence for an accumulation of Th1 T cells in the Eμ-TCL1 mouse model of CLL. As TBET (encoded by Tbx21) is a crucial transcription factor for Th1 polarization, we generated Tbx21−/− bone marrow chimeraic mice which showed a lower number of IFNγ-producing Th1 T cells, and used them for adoptive transfer of Eμ-TCL1 leukaemia. Disease development in these mice was, however, comparable to that in wild-type controls, excluding a major role for TBET-expressing Th1 cells in Eμ-TCL1 leukaemia. Collectively, our data highlight that Th1 T cells accumulate in CLL but reducing their number has no impact on disease development.

Keywords: CLL, CD4+ T cells, Th1, IFNγ, TBET.

Introduction
Chronic lymphocytic leukaemia (CLL) is a common leukaemia, which results in the accumulation of mature CD5+ B cells in peripheral blood (PB) and lymphoid organs, such as bone marrow (BM) and lymph nodes (Yosifov et al., 2019). Recently, novel, targeted therapeutic approaches including ibrutinib, venetoclax and idelalisib have dramatically changed treatment regimens (Yosifov et al., 2019). In addition to targeting the malignant cells directly, an impact of these inhibitors on non-malignant immune cells, including T cells, was reported (Dubovsky et al., 2013; Kohrt et al., 2014; Hanna et al., 2019a). As these cells are known to play a role during CLL progression, it is important to fully characterize their distribution and function in CLL. Thorough investigations of CD8+ T cells have been performed by us and others (Riches et al., 2010; Hanna et al., 2019b); however, a profound understanding of CD4+ T cells in CLL is still lacking.

After antigen recognition, naive CD4+ T cells get activated, expand clonally and differentiate into effector T cells (Jelley-Gibbs et al., 2008). Subsequently, CD4+ T cells orchestrate responses of CD8+ T cells and B cells against pathogens (Parkar, 1993; Laidlaw et al., 2016).

CD4+ T cells are a heterogeneous population of cells, broadly divided into conventional (cCD4+) and regulatory (Treg) subsets. Previous studies showed Tregs to be enriched in CLL, although their depletion caused only limited changes in CLL progression in a mouse model of CLL (D’Arena et al., 2011; Hanna et al., 2019a).

T-helper cells are categorized into three major subsets, namely Th1, Th2 and Th17 cells, which are characterized by their specific production of cytokines and functions (Jelley-Gibbs et al., 2008). Th1 cells are defined by their production of interferon gamma (IFNγ) (Mosmann et al., 1986; Jelley-Gibbs et al., 2008), the expression of their master transcription factor T-box transcription factor 21 (Tbx21, TBET)
(Szabo et al., 2000), as well as their surface expression of CXC motif receptor 3 (CXCR3), which is commonly used for their identification in PB (Bonecchi et al., 1998). This cell type aids the elimination of intracellular pathogens, by activating macrophages in an IFNγ-dependent manner (Suzuki et al., 1988).

Th2 cells are involved in the clearance of extracellular parasites, such as helminths (Jelley-Gibbs et al., 2008). Interleukin-4 (IL-4), the key cytokine of Th2 cells, initiates the immunoglobulin (Ig) class switching of B cells to IgE, which causes mast cell degranulation (Punnonen et al., 1997; Galli & Tsai, 2012). Next to their secretion of CC motif chemokine receptor 6 (CCL6) have been useful for identification of Th17 cells (Acosta-Rodriguez et al., 2007). Characterizing the different CD4+ T-helper cell subsets in CLL so far has resulted in divergent results as both a Th1- as well as a Th2-enriched environment were proposed. Some studies showed a Th1 dominance upon analyzing patient-derived samples (Podhorecka et al., 2002; Palma et al., 2017), although other studies using CLL patient samples and mouse models suggested a dominance of Th2-like T cells (Rossmann et al., 2002; Gorgun et al., 2005; Buggins et al., 2007; McClanahan et al., 2015). Focusing on the function of CD4+ T cells in CLL, it was shown that CD4+ T cells were necessary to induce CLL cell proliferation in a xenograft mouse model (Bagnara et al., 2011). Additionally, CD4+ Th1-like T cells were shown to activate autologous CLL cells and induce CLL cell proliferation in an antigen- and IFNγ-dependent manner (Os et al., 2013; Burgler et al., 2015). Results using different ablative strategies of CD4+ T cells in CLL remained inconclusive thus far. On the one hand, using genetically modified GK5 mice, which lack all CD4+ T cells, a faster disease progression was observed (Kocher et al., 2016). On the other hand, CD4+ T cell depletion by exogenous antibody treatment revealed no difference in CLL progression (Hanna et al., 2019a). In sum, the function of CD4+ T cells during CLL progression still needs to be thoroughly investigated as it might be highly dependent on disease burden and number of remaining CD4+ T cells after ablative strategies. By an in-depth analysis of surface markers, master transcriptional regulators and cytokines in CD4+ T cells, we aimed at identifying the dominant T-helper subset in CLL patient samples as well as mice with CLL-like disease. Furthermore, the influence of Th1 T cells on CLL development was evaluated.

### Methods

#### Patient samples

Patient samples were obtained after approval of the study protocols by local ethics committees from the Department of Internal Medicine III of Ulm University and the Hospital Clinic of Barcelona according to the declaration of Helsinki, and after obtaining informed consent of patients. Patients met standard diagnosis criteria for CLL. Patient characteristics such as age, mutational state and Binet stage are depicted separately (Tables SI and SII). Healthy, age-matched controls were obtained from Biomex GmbH (Heidelberg, Germany) after informed consent. PB was drawn using tubes coated with ethylenediaminetetraacetic acid (EDTA) (Sarstedt, Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation. If necessary, PBMCs were viable frozen and when needed, frozen PBMCs were thawed and rested for three hours until further processing. Cytomegalovirus (CMV) immunoglobulin G (IgG) ELISA (Virotech Diagnostics, Rüsselsheim, Germany) was performed according to the manufacturer’s protocol using serum samples.

#### Mice and tumour models

Adoptive transfer was performed by i.p. or i.v transplantation of 1–2 × 10⁷ leukaemic Eq-TCL1 splenocytes into C57BL/6 N or J wild-type (WT) animals. Splenocytes were enriched in CD19+ cells using EasySep™ Mouse Pan-B Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada) yielding a purity above 95% of CD5+ CD19+ cells.

Bone marrow chimaeras were generated as previously described (Öztürk et al., 2019). In brief, chimaeras were generated by i.v. injection of 5 × 10⁶ BM cells into WT mice two days after their gamma-irradiation with two cycles of 500 cGy. For single chimaeras, BM cells of WT or Tbx21tm1cl/cam (Tbx21−/−) (Jackson Laboratory, Bar Harbor, ME) (Finotto et al., 2002) animals were used. For the generation of mixed chimaeras, WT and Tbx21−/− cells were simultaneously transferred in a 1:1 ratio. Reconstitution of the haematopoietic system in these mice was obtained eight to twelve weeks after BM transfer as monitored by flow cytometry. One week later, mice were i.v. injected with 1 × 10⁷ Eq-TCL1 tumour cells.

All animal experiments were carried out according to institutional and governmental guidelines approved by the local authorities (Regierungspräsidium Karlsruhe, permit numbers: G25/16, G36/14, G98/16, G123/14, and Regierungspräsidium Tübingen TVA 1124). Eq-TCL1 mice (C. Croce, OH, USA) were kept under specific pathogen-free conditions on a pure C57BL/6 N or J background at the central animal facility of the German Cancer Research Center (DKFZ).
**Flow cytometry**

A detailed description of flow cytometric assays and gating strategies is given in the Supplementary material. In case of a bimodal distribution of signal, the percentage of positive cells is depicted, whereas signals with unimodal distribution are displayed as median fluorescence intensities (MFI). Normalized MFIs (nMFIs) were calculated by subtracting the MFI of the respective fluorescence-minus-one controls.

**Statistical analysis**

Data were acquired using a BD FACS Canto II, BD LSR II or BD LSR Fortessa (BD Biosciences, Heidelberg, Germany) fluorescence-activated cell sorting (FACS) analyzer. Flow cytometric data were analyzed using FlowJo X 10.0.7 software (FlowJo, Ashland, OR, USA).

Data were analyzed using Prism 7 GraphPad software (GraphPad Software, La Jolla, CA, USA). Samples of different groups were compared using non-parametric Mann–Whitney testing. Correlations of parameters with the CLL tumour burden were analyzed using Spearman’s correlation. The respective statistical tests are mentioned in the figure legends. Values of \( P < 0.05 \) were considered statistically significant. All graphs show means ± SEM, unless otherwise indicated.

**Results**

**T cells are enriched in blood of CLL patients**

Elevated numbers of blood T cells in CLL patients in comparison to healthy controls (HCs) were described already more than 40 years ago (Catovsky et al., 1974). Furthermore, a decrease of the blood CD4:CD8 ratio, mainly caused by an increase in CD8\(^+\) T cells, is well established in CLL (Herrmann et al., 1982; Platsoucas et al., 1982). Using a cohort of 21 treatment-naive CLL patients with varying CLL burden from 6,200 to 134,700 CLL cells per μl blood and 19 age-matched HC, an increase in absolute T cell numbers in blood was confirmed (Fig 1A, flow cytometry gating strategy Figure S1A). Moreover, the CD4:CD8 ratio (calculated from the total number of CD3\(^+\) T cells) trended to be reduced in CLL patients (Fig 1B). This analysis further showed that the

Fig 1. CD4\(^+\) T cell counts correlate with patients’ CLL burden. Whole blood samples of age-matched healthy controls (HC; \( n = 19 \)) and CLL patients (CLL; \( n = 21 \)) were analyzed by flow cytometry. (A) Counts of total CD3\(^+\) and CD3\(^+\) CD4\(^+\) T cells per μl blood of HCs and CLL. (B) Representative graphs (top) and quantification (bottom) of CD4:CD8 ratio calculated out of total CD3\(^+\) T cells of HCs and CLL patients. (C) Spearman’s correlation of total CD3\(^+\) T cell and CD3\(^+\) CD4\(^+\) T cell counts per μl blood with CLL cell counts (defined as number of non-CD3 lymphocytes) per μl blood. All graphs show mean ± SEM. Statistical analysis was performed using the Mann–Whitney test. **, \( P < 0.01 \). CLL, Chronic lymphocytic leukaemia; HC, Healthy control. [Colour figure can be viewed at wileyonlinelibrary.com]
number of total T cells as well as CD4+ T cells positively correlated with patients’ CLL cell counts in the blood (Fig 1C).

Besides the two well-known CD4+ and CD8+ T cell subsets, the existence of CD4+ CD8+ double negative (DN) and CD4+ CD8+ double positive (DP) populations in the blood have been described (Ghia et al., 2007; Martina et al., 2015). In the analyzed CLL patient cohort, we detected an enrichment of DN T cells in comparison to HCs (Figure S1B). Moreover, the number of DN T cells was positively correlating with patients’ CLL burden (Figure S1C, D), whereas DP T cells were less abundant and no significant differences between patients and controls were observed (Figure S1E, F). Thus, no significant differences between patients and controls were observed (Figure S1E, F), and numbers of NKT cells were about 10-fold lower for HCs and 24-fold lower for CLL than numbers of DN T cells (Figure S1B, E), suggesting a real increase in DN T cells in CLL.

Taken together, CLL is associated with an accumulation of T cells in the blood which correlates with CLL burden. Besides increased numbers of CD4+ and CD8+ T cells, an enrichment of CD4+ CD8+ DN T cells was seen in CLL.

**Th1-like T cells accumulate in the blood of CLL patients**

To analyze the distribution of T-helper subsets in peripheral blood (PB) of CLL patients, flow cytometry was performed. CD4+ T-helper-like subsets were defined according to their surface expression of CXCR3 and CCR6 into CXCR3+ CCR6− Th1-like, CXCR3− CCR6+ Th2-like, CXCR3− CCR6− Th17-like T cells, and CXCR3+ CCR6+ CD4+ T cells.
CD4⁺ T cells of CLL patients show hallmarks of Th1 T cells

As T-helper-like subsets, defined by surface markers, contain cells producing key cytokines of other T-helper subsets (Kim et al., 2001), analyses of T-helper transcriptional master regulators as well as expression of key cytokines were performed. Accordingly, Th1 T cells were defined by their expression of TBET (Szabo et al., 2000) and their IFNγ secretion (Mossman et al., 1986; Jelley-Gibbs et al., 2008), and Th2 T cells by GATA3 expression (Kurata et al., 1999) and IL-4 secretion (Jelley-Gibbs et al., 2008), respectively.

To review the data retrieved by surface marker analyses, ex vivo mitogen stimulation of T cells with PMA/ionomycin was performed. CD4⁺ T cells of CLL patients expressed IFNγ to a higher extent in comparison to HCs, whereas no difference in IL-4 production was seen (Fig 3A–B).

Further, analyses of master regulators of T-helper cell differentiation of FoxP3⁻ conventional CD4⁺ (cCD4⁺) cells were performed, to exclude Tregs, which were shown to express T-helper cell-specific transcription factors (Koch et al., 2009). CLL patient-derived cCD4⁺ T cells expressed more TBET in comparison to HCs (Fig 3C), whereas no difference in the expression of GATA3 was evident (Fig 3D). As suggested by literature, expression of TBET and GATA3 was particularly high in CD45RO⁺ cCD4⁺ memory T cells (Figure S2A–B) (Knox et al., 2014). Of note, CLL-derived memory cCD4⁺ T cells contained a higher frequency of TBET-expressing cells in comparison to HC (Figure S2A), whereas no difference in GATA3 was seen (Figure S2B).

In sum, Th1 T cells are the dominating T-helper subset in human CLL based on analyses of key cytokines as well as master transcription factors.

Patient’s T-helper cells are stable during disease progression

To investigate dynamic changes of T-helper cells in individual patients with increasing CLL cell counts in blood (Fig 4A), the expression of IFNγ and IL-4, as well as TBET and GATA3 were analyzed in CD4⁺ T cells from blood of six treatment-naïve CLL patients over a time span of approximately five years. This revealed rather stable frequencies of Th1 and Th2 subsets in individual patients over time with only one case (CLL149) out of six patients that showed an increase in the percentage of CD4⁺ T cells expressing IFNγ, IL-4 or TBET at one of the analyzed time points (Fig 4B–D). Given that CLL is known to be associated with immunodeficiency, this could be easily explained by a transient viral infection of this patient, even though we have no clinical information on that (Ravandi & O’Brien, 2006; Hanna et al., 2019b). In accordance, the master transcriptional regulators TBET (Fig 4D) and GATA3 (Fig 4E) revealed similar results, suggesting that frequencies of T-helper subsets are overall stable during disease course of CLL patients, lacking clinical signs of progressive disease.

Th1 T cells accumulate in the TCL1 AT mouse model of CLL over time

The Eμ-TCL1 mouse model of CLL is widely used to analyze effects of immune cells on disease progression (Hanna et al., 2016). Upon syngeneic transplantation of malignant splenocytes of Eμ-TCL1 mice (TCL1 AT), the recipient mice develop a CLL-like leukaemic disease after four to six weeks with an accumulation of malignant B cells mainly in the spleen. To track CD4⁺ T cell polarization associated with CLL-like disease in these mice, splenic T-helper cell subsets were analyzed. Percentages of TBET-positive cells as well as the normalized median fluorescence intensity (nMFI) of GATA3 in cCD4⁺ T cells were higher in leukaemic mice in comparison to WT controls (Fig 5A–B, flow cytometry gating strategy Figure S3A–B). Of note, only low frequencies of TBET⁺ as well as GATA3⁺ cCD4⁺ T cells were detected in untransplanted WT animals (Fig 5A, Figure S3C). Comparing the percentages of TBET⁺ to GATA3⁺ cCD4⁺ T cells revealed an about threefold higher frequency of TBET-expressing cells (Fig 5A, Figure S3C). These data suggest that Th1 T cells dominate in the spleen of mice with CLL-like disease.

To investigate whether T-helper cell polarization is dynamic in this mouse model of CLL, the expression of IFNγ in cCD4⁺ T cells was analyzed at different time points after adoptive transfer of leukaemia. Remarkably, the percentage of IFNγ-producing cCD4⁺ T cells following ex vivo PMA/ionomycin stimulation was higher at weeks six and eight after adoptive

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transfer of leukaemic cells in comparison to WT controls with about 70% of cCD4+ T cells producing IFNγ at week 8 in TCL1 AT mice (Fig 5C). No difference in the expression of IFNγ was evident at week 2 after TCL1 AT (Fig 5C).

In conclusion, Th1 CD4+ T cells are the dominating T-helper subset in the TCL1 AT model of CLL, based on expression of master transcriptional regulators. Furthermore, the secretion of IFNγ by cCD4+ T cells increases during disease progression.

Reduced frequency of IFNγ-expressing Th1 T cells in Tbx21−/− chimaera does not affect CLL development

To investigate whether Th1 T cells have a pro- or antitumoral effect on CLL development, Tbx21-deficient mice were used. Previously, CD4+ T cells of Tbx21−/− mice were shown not to polarize into IFNγ-producing Th1 T cells, resulting in reduced clearance of intracellular pathogens (Szabo et al., 2002). Of note, cytotoxic CD8+ T cell function was shown not to be altered due to deficiency of Tbx21, whereas IFNγ-production by NKT cells was reduced (Szabo et al., 2002). To avoid rejection of TCL1 leukaemia cells after their transplantation in Tbx21−/− mice, as previously shown in comparable approaches (Öztürk et al., 2019), Tbx21−/− BM chimaeras and the respective WT control animals were generated. In addition to this approach, mixed chimaeras were generated by transplantation of a 1:1 mixture of WT and Tbx21−/− BM cells to distinguish direct effects of Tbx21-deficiency in CD4+ T cells on IFNγ production from indirect effects that might exist in a Tbx21-deficient haematopoietic

Fig 3. Frequency of Th1 CD4+ T cells is higher in blood of CLL patients compared to healthy controls. Peripheral blood mononuclear cells (PBMCs) of healthy controls (HC) and CLL patients (CLL) were analyzed by flow cytometry. (A, B) PBMCs were stimulated ex vivo with PMA/ionomycin and production of (A) IFNγ and (B) IL-4 in CD4+ T cells of HCs (n = 7) and CLL (n = 24) were assessed by intracellular cytokine staining. Representative graphs (top) and percentages (bottom) of cytokine-producing cells. (C, D) Expression of master transcription factors for Th1 and Th2 polarization of HCs (n = 11) and CLL (n = 21–22) were analyzed. Representative graph (left) and percentages of transcription factor-expressing cells out of FoxP3− conventional CD4+ (cCD4+) T cells (right) are shown. (C) Expression of TBET as marker for Th1, and (D) GATA3 for Th2 polarization, respectively. All graphs show mean ± SEM. Statistical analysis was performed using the Mann–Whitney test. *, P < 0.05. CLL, Chronic lymphocytic leukaemia; HC, Healthy control; SSC-A, side scatter area. [Colour figure can be viewed at wileyonlinelibrary.com]
microenvironment. Upon reconstitution of the haematopoietic system about eight weeks after BM cell transfer, chimaeric mice were transplanted with leukaemic Eµ-TCL1 splenocytes. As expected, Tbx21−/−/C0 cCD4+ T cells showed an approximately twofold decreased production of IFNγ in comparison to WT cells in the mixed chimaeras, proving a direct Tbx21-regulated reduction of Th1 T cells (Fig 6A).

Using single BM chimaeras, in which a minor fraction of host-derived Tbx21-proficient CD45/2/C12 WT CD4+ T cells was remaining (Figure S4A), the TBET expression of CD45/2/cCD4+ T cells was almost absent in Tbx21−/−/C0 chimaeras, resulting in an about twofold reduced production of IFNγ (Fig 6B–C). Of note, no concomitant increase in nMFI and percentage of GATA3 expression of cCD4+ T cells was seen in Tbx21−/−/C0 chimaeras (Fig 6D, Figure S4B).

To investigate whether a reduction in Th1 T cells in Tbx21−/−/− chimaeras impacts on CLL progression, organ weight of spleen and liver, as a measure of disease burden, as well as percentage of CD5+ CD19+ CLL-like cells in lymphoid organs were analyzed. Intriguingly, neither spleen and liver weight nor tumour burden in lymphoid organs, such as spleen, lymph node (LN) and BM, were different in Tbx21−/−/− compared to WT chimaeras (Fig 6E–F). To exclude that major alterations of CD8+ T cells could mask effects on CLL burden, the effector function of CD45/2+ CD8+ T cells was investigated. Of importance, a significantly lower IFNγ but not granzyme B (GzmB) production, as well as lower CD107a surface localization, as surrogate for degranulation capacity, was observed in total CD8+ T cells as well as in CD8+ CD44+ CD127− effector T cells of Tbx21−/−/− compared to WT chimaeras (Figure S4C–F) (Hanna et al., 2019b).

In conclusion, Tbx21-deficiency reduced the frequency of IFNγ-producing Th1 T cells in CLL-bearing BM chimaeras. Nevertheless, reduction in Th1 T cells did neither speed up nor slow down CLL progression.

Discussion

Alterations of T cell numbers and their subset distribution have been described for CLL patients in comparison to healthy controls (HC). Among these, increased numbers of blood T cells as well as a decrease of the CD4:CD8 ratio...
mainly due to higher numbers of CD8$^+$ T cells were reported, results that were confirmed by our study (Catovsky et al., 1974; Herrmann et al., 1982; Platsoucas et al., 1982; Hanna et al., 2019b). The role of CD4$^+$ helper T cells in CLL is controversial, as these cells were shown to support survival and growth of CLL cells, but are also known to support adaptive immune control of tumours (Bagnara et al., 2011; Os et al., 2013; Burgler et al., 2015).

Recently, different approaches, which aimed to elucidate the role of CD4$^+$ T cells in CLL progression, were published. Kocher et al. used a genetically modified mouse model, which lacks CD4$^+$ T cells due to its intrinsic αCD4-antibody production (GK5 mice), as recipient for Flt3-TCL1 leukaemic cells and showed a faster CLL progression in the absence of CD4$^+$ T cells (Kocher et al., 2016). In contrast, using less efficient depletion of cells by exogenous αCD4-antibody

Fig 5. Th1 CD4$^+$ T cells accumulate in spleen of TCL1 AT mice. Splenocytes of untransplanted wild-type (WT) mice and animals, which were transplanted i.p. with 1–2 x 10$^7$ leukaemic cells (TCL1 AT), were analyzed by flow cytometry. (A) Representative graphs (top) and percentages (bottom) of TBET-expressing cells, as well as (B) nMFI of GATA3 out of FoxP3$^+$ conventional CD4$^+$ (cCD4$^+$) T cells of WT ($n = 4$) and TCL1 AT ($n = 10$) animals four weeks after adoptive transfer of leukaemic cells. (C) Splenocytes were stimulated ex vivo for 6 h with PMA/ionomycin and intracellular flow cytometry was performed. Percentages of IFN$^+$ cells out of cCD4$^+$ T cells of WT animals or at indicated time points after TCL1 AT. Representative graph (left) at week 8 after TCL1 AT. All graphs show mean ± SEM. Statistical analysis was performed using the Mann–Whitney test. *, $P < 0.05$; **, $P < 0.01$. nMFI, normalized median fluorescence intensity. SSC-A, side scatter area. [Colour figure can be viewed at wileyonlinelibrary.com]
injections, no difference in Δμ-TCL1 leukaemia development was seen compared to control-antibody-treated mice (Hanna et al., 2019b). Besides these systemic approaches that aimed at elucidating the function of CD4<sup>+</sup> T cells in CLL, many descriptive studies are available, which intend to characterize T-helper cell subsets. So far, the results of these studies have been controversial and therefore, inconclusive. Utilizing gene expression profiling as well as ex vivo IL-4 production following mitogen stimulation of patient-derived CD4<sup>+</sup> T cells, a dominance of Th2 T cells was suggested by some studies (Rossmann et al., 2002; Gorgun et al., 2005). Of note, McClanahan et al. reported no difference in IFN<sub>γ</sub> production of CD4<sup>+</sup> T cells, whereas an increased frequency of IL-4<sup>+</sup> CD4<sup>+</sup> T cells was seen in ageing Δμ-TCL1 mice (McClanahan et al., 2015). Of importance, the frequency of IFN<sub>γ</sub>-producing Th1 T cells observed in this study was approximately threefold higher than that of IL-4-expressing Th2 T cells (McClanahan et al., 2015). Along this line, a Th1 dominance was suggested by surface marker analysis and cytokine production after ex vivo stimulation of CLL patient-derived T cells (Podhorecka et al., 2002; Palma et al., 2017). Analyzing serum cytokine levels, an increased concentration of IFN<sub>γ</sub> but no differences in IL-4 levels were reported in CLL patients in comparison to HCs (Yan et al., 2011). These results are supported by our data, showing a larger fraction of Th1-like CD4<sup>+</sup> T cells, based on expression of surface markers CXCR3 and CCR6, in the blood of CLL patients compared to HCs, as previously seen by Palma et al. using the same marker combination (Palma et al., 2017). But as that cell surface expression of these chemokine receptors has been shown not to accurately define T-helper subsets (Kim et al., 2001), we subsequently analyzed cytokine production and expression of master transcriptional regulators in CLL blood samples as well as splenocytes of leukemic TCL1 AT mice, which revealed an enrichment of Th1 CD4<sup>+</sup> T cells in CLL. Observations in patients during their disease course suggested an early skewing towards a Th1 milieu, as no differences in the frequency of T-helper subsets were seen over several years with increasing CLL burden. Taken together, the microenvironment of CLL is enriched by Th1 CD4<sup>+</sup> T cells, and their frequencies are stable over time.

Apart from the well-known CD4<sup>+</sup> CD8<sup>+</sup> and CD4<sup>+</sup> CD8<sup>+</sup> T cell subsets, the existence of CD4<sup>+</sup> CD8<sup>-</sup> DN T cells as well as CD4<sup>+</sup> CD8<sup>-</sup> DP T cells in PB was described (Ghia et al., 2007; Martina et al., 2015). The latter population was shown to increase with age (Ghia et al., 2007). DN T cells were found to poorly respond to T cell receptor stimulation with an increase in proliferation and IL-2 secretion (Davignon et al., 1988; Martina et al., 2015). Two concurrent hypotheses have been proposed for the accumulation of DN T cells. Firstly, an impaired Fas-mediated clearance of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells causes the downregulation of the CD4 or CD8 co-receptors and ultimately the accumulation of DN T cells (Landolfi et al., 1993; Martina et al., 2015). Secondly, the normally rare, pre-existing subset of DN T cells is thought to accumulate due to impaired Fas-mediated apoptosis in the periphery (Mohamood et al., 2008; Martina et al., 2015). Analysis of a CLL patient cohort in our study showed an increase in DN T cells, whereas no alterations in DP T cells was seen, indicating that Fas-mediated clearance of T cells could be altered in CLL.

Th1 CD4<sup>+</sup> T cells are thought to indirectly mediate immune control of tumours by their secretion of IFN<sub>γ</sub>, which shapes and directs cytotoxic activity of other immune cells such as macrophages (Haabeth et al., 2011). In CLL, IFN<sub>γ</sub> was shown to inhibit apoptosis of CLL cells in vitro (Buschle et al., 1993). Furthermore, proliferation of patient-derived CLL cells in vitro and in mouse models was induced after interaction with autologous Th1-like T cells that recognize CLL antigens and express CXCR3, Tbet and IFN<sub>γ</sub> (Os et al., 2013; Burgler et al., 2015). Moreover, in a xenograft mouse model of CLL, Th1-like IFN<sub>γ</sub>-expressing CD4<sup>+</sup> T cells guided the differentiation of CLL cells into antibody-secreting plasma cells (Patten et al., 2016). Altogether, these data suggest that IFN<sub>γ</sub>, among other cytokines, supports CLL cells. This is in line with the observation that CLL cells are dependent on extracellular stimuli that trigger B cell receptor, Toll-like receptor or cytokine receptor signalling (Hanna et al., 2017; Gimenez et al., 2019). In contrary, we recently showed that neutralizing IFN<sub>γ</sub> via injections of blocking antibodies in the TCL1 AT mouse model resulted in faster leukaemia progression in these mice (Hanna et al., 2019b). As IFN<sub>γ</sub> is a key cytokine for the activation of cytotoxic T cells, this previous study highlights the relevance of adaptive immune control in CLL. To investigate the role of IFN<sub>γ</sub>-expressing Th1 T cells in CLL, we used Tbx21<sup>−/−</sup> BM chimaeras. Tbx21 was shown to be essential for the generation of Th1 T cells (Szabo et al., 2000), and Tbx21<sup>−/−</sup> CD4<sup>+</sup> T cells fail to polarize into Th1 cells (Szabo et al., 2002). Using Tbx21<sup>−/−</sup> BM chimaeras, a reduction of Th1 T cells was seen in this study, which did not result in any alterations of CLL progression. Of note, a minor remaining fraction of Tbx21-proficient WT CD4<sup>+</sup> T cells as well as a slightly reduced functional capacity of CD8<sup>+</sup> T cells were observed in these mice. These results suggest that Th1 cells and their produced IFN<sub>γ</sub> have no impact on CLL development. Like several other immune cell types, including NKT cells, CD8<sup>+</sup> T cells and antigen-presenting cells are known to secrete IFN<sub>γ</sub> (Ohteki et al., 1999; Pahl & Cerwenka, 2017; Hanna et al., 2019c), and a lack of Th1 cell-derived IFN<sub>γ</sub> is not sufficient to have an impact on adaptive immune control of CLL, as observed in mice with systemic depletion of IFN<sub>γ</sub> (Hanna et al., 2019c).

In summary, Th1 T cells accumulate in human CLL and the TCL1 AT mouse model, although they likely do not influence disease progression. To further explore whether this is also true for total CD4<sup>+</sup> T cells or other CD4<sup>+</sup> subsets, such as T-follicular helper (THF) cells, subset-defined approaches need to be undertaken to clarify the role of the heterogeneous population of CD4<sup>+</sup> T cells in the pathogenesis of CLL.
(A) Isotype
WT cells
Tbx21−/− cells

(B) WT chimaera
Tbx21−/− chimaera

(C) WT chimaera
Tbx21−/− chimaera

(D) WT chimaera
Tbx21−/− chimaera

(E) WT chimaera
Tbx21−/− chimaera

(F) WT chimaera
Tbx21−/− chimaera

(G) WT chimaera
Tbx21−/− chimaera

Organ weight [g]
Spleen, Liver

CLL cells per spleen x10^6

% CD5+ CD19+ CLL cells of CD45+ cells

Spleen, LN, BM
Fig 6. Thbx21-deficient mice have lower numbers of IFNγ-expressing Th1 cells but show no difference in CLL development compared to wild-type (WT) mice. WT mice were lethally irradiated, followed by i.v. transplantation of 5 × 10⁶ bone marrow (BM) cells. Upon reconstitution of the haematopoietic system 8–12 weeks after BM transfer, mice were i.v. transplanted with 1–2 × 10⁷ leukaemic Eμ-TCL1 tumour cells. (A) Mixed chimaeras were generated by transplantation of WT and Thbx21–/– BM cells at a 1:1 ratio. Six weeks after adoptive transfer of TCL1 leukaemia, splenocytes were stimulated ex vivo for 6 h with PMA/ionomycin. Representative graph (top) and percentages (bottom) of IFNγ+ cells out of WT and Thbx21–/– conventional FoxP3– CD4+ (cCD4+) T cells in mixed chimaeras are shown. Statistical analysis was performed using paired Student’s t-test. (B–G) Single chimaeras were generated by transplantation of WT or Thbx21–/– BM cells. Upon reconstitution of the haematopoietic system after 12 weeks, chimaeras were transplanted with TCL1 leukaemia cells and analyzed after six weeks. (B) Representative graph (top) and percentages of TBET+ cells (bottom) out of cCD4+ T cells are shown. (C) Splenocytes were stimulated ex vivo for 6 h with PMA/ionomycin and intracellular flow cytometry was performed. Percentages of IFNγ+ cells out of cCD4+ T cells of WT and Thbx21–/– chimaeras were analyzed. (D) nMFI of GATA3 of cCD4+ T cells (right) were analyzed by intracellular flow cytometry. A representative graph is depicted on the left. (E) Organ weights of spleen and liver were assessed as surrogate of CLL burden of WT and Thbx21–/– chimaeras. (F) Total number of CLL cells per spleen, as well as (G) frequency of CD5+ CD19+ CLL cells out of CD45+ haematopoietic cells in spleen, lymph node (LN) and bone marrow (BM) of WT and Thbx21–/– chimaeras was analyzed. All graphs show mean ± SEM. Statistical analysis was performed using the Mann–Whitney test unless otherwise indicated. *, P < 0.05; **, P < 0.01; ***, P < 0.001. nMFI, normalized median fluorescence intensity. CLL, Chronic lymphocytic leukaemia. SSC-A, side scatter area. [Colour figure can be viewed at wileyonlinelibrary.com]

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Author contributions
PMR designed the study, performed experiments, analyzed and interpreted data, prepared figures, and wrote the manuscript. BSH designed the study, performed experiments, analyzed and interpreted data. SO performed experiments and interpreted data. RS, LLC, HY and AS performed experiments. DC and StSt provided clinical samples and information. PL critically advised the study and reviewed the manuscript. MS designed and supervised the study, interpreted data, and wrote the manuscript.

Competing interests
The authors declare that they have no competing interests.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Clinical information for whole blood samples of CLL patients and healthy controls.
Table SII. Clinical information for transcription factor and cytokine analyses of CLL patients and healthy controls.
Table SIII. List of flow cytometry antibodies.
Table SIV. Clinical information for transcription factor and cytokine analyses of CLL patients and healthy controls.

Fig S1. T cells accumulate in the spleen of healthy controls.
Fig S2. Frequency of Th1 CD4+ T cells is higher in blood of CLL patients compared to healthy controls.
Fig S3. Th1 CD4+ T cells accumulate in the spleen of TCL1 AT mice.
Fig S4. Thbx21-deficiency causes a reduction of CD8+ T cell function.

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