ORIGINAL ARTICLE

Fludarabine modulates composition and function of the T cell pool in patients with chronic lymphocytic leukaemia

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Abstract The combination of cytotoxic treatment with strategies for immune activation represents an attractive strategy for tumour therapy. Following reduction of high tumour burden by effective cytotoxic agents, two major immune-stimulating approaches are being pursued. First, innate immunity can be activated by monoclonal antibodies triggering antibody-dependent cellular cytotoxicity. Second, tumour-specific T cell responses can be generated by immunization of patients with peptides derived from tumour antigens and infused in soluble form or loaded onto dendritic cells. The choice of cytotoxic agents for such combinatory regimens is crucial since most substances such as fludarabine are considered immunosuppressive while others such as cyclophosphamide can have immunostimulatory activity. We tested in this study whether fludarabine and/or cyclophosphamide, which represent a very effective treatment regimen for chronic lymphocytic leukaemia, would interfere with a therapeutic strategy of T cell activation. Analysis of peripheral blood samples from patients prior and during fludarabine/cyclophosphamide therapy revealed rapid and sustained reduction of tumour cells but also of CD4$^+$ and CD8$^+$ T cells. This correlated with a significant cytotoxic activity of fludarabine/cyclophosphamide on T cells in vitro. Unexpectedly, T cells surviving fludarabine/cyclophosphamide treatment in vitro had a more mature phenotype, while fludarabine-treated T cells were significantly more responsive to mitogenic stimulation than their untreated counterparts and showed a shift towards T_H1 cytokine secretion. In conclusion, fludarabine/cyclophosphamide therapy though inducing significant and relevant T cell depletion seems to generate a micromilieu suitable for subsequent T cell activation.

Keywords Fludarabine · CLL · Immunomodulation · T cells

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Introduction

In general, the success or failure of cancer immunotherapy depends on whether efficient stimulation of T cells and subsequent establishment of an activated, tumour-specific memory T cell pool is accomplished or not. While our knowledge of the biology of tumour cells, antigen-presenting cells and their complex interactions with autologous T cells increases, the challenges regarding clinical efficacy of immunotherapy remain to be resolved [1–6]. Among them are the rather low frequency of tumour antigen-specific T cells, their limited life span and their loss of cytolytic activity in vaccination studies [7]. It can also be expected that the efficiency of T cell activation following vaccination is generally reduced in patients with intrinsic T cell deficiencies.

In chronic lymphocytic leukaemia (CLL), changes within the T cell compartment comprise abnormal increase in absolute CD4^{+} and CD8^{+} T cell numbers with abnormal phenotype, such as increase in death receptor expression [8], loss of costimulatory molecules necessary for stimulation of antigen-presenting cells [9], abnormal cytokine/receptor profile [10], impaired formation of the immune synapse [11] as well as accumulation of regulatory T cells (Treg) [12, 13]. All these alterations might contribute to the decreased responsiveness of T cells to antigenic stimulation and to the hypogammaglobulinaemia observed in CLL patients, making them highly susceptible to severe opportunistic infections [14]. In addition, it might allow unhindered expansion of the tumour clone in phases of disease progression or tumour relapse following therapy.

A combination of two agents, the nucleoside analogue fludarabine, and cyclophosphamide, a DNA-alkylating agent, has been shown to be a highly effective treatment for CLL patients [15–17]. Efficacy can even be improved by combining fludarabine/cyclophosphamide with additional therapeutics like mitoxantron [18] or monoclonal antibodies like rituximab [19–21]. It has already been shown that cyclophosphamide, beside being cytoreductive, is able to enhance lymphocyte reconstitution and immune responses (for review [3, 22]), whereas fludarabine is routinely administered in the setting of allogeneic stem cell transplantation and leads to prolonged immunosuppression [23]. The aim of our study was to characterize activation status, subset distribution and functions of the T cell compartment from CLL patients after treatment with fludarabine, cyclophosphamide or both.

Materials and methods

Patients

The study was conducted according to the Declaration of Helsinki. After obtaining informed patient consent, untreated CLL patients seen at the Department of Haematology and Oncology, University Hospital Salzburg, Austria between January 2005 and April 2010 were included in this study. Peripheral blood mononuclear cells (PBMCs) were collected in heparinized or EDTA-coated tubes during routine examinations. CLL was defined by clinical criteria as well as by cellular morphology and the coexpression of CD19, CD5, and CD23 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Staging was performed according to modified Rai classification [24]. Characteristics of the patients studied are presented in Table 1. For those experiments where not all patients were included, the patient samples were unselected.

Table 1 Patient characteristics

Parameters	No. of patients (%)
Total number	55 (100)
Sex	
Male	35 (64)
Female	20 (36)
Age (years)	
Median	72
Range	47–94
Duration of disease (years)	
Median	5.7
Range	1.75–27.1
Rai stage	
Low (0)	23 (42)
Intermediate (I/II)	27 (49)
High (III/IV)	5 (9)
Molecular risk parameters	
Unmutated Ig VH	15 (29) (nda: 4)
Unfavourable genetic aberration(s) (del17p, 11q)	2 (4) (nda: 1)
CD38 + B-CLL ($\geq$30%)	15 (29) (nda: 3)

B-CLL B cell chronic lymphocytic leukaemia, *Ig* immunoglobulin, *VH* variable heavy chain, *nda* no data available

Purification of PBMC and cell culture

Peripheral blood mononuclear cells were separated by density centrifugation. Fresh blood samples were diluted 1:1 with RPMI 1640 (PAA Laboratories, Pasching, Austria) and layered over Biocoll separating solution (Biochrom AG, Berlin, Germany). The opaque layer on top of the Biocoll-containing PBMCs was collected, washed once with phosphate-buffered saline (PBS) and resuspended in complete medium [RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10% fetal calf serum (FCS)]. PBMCs were then incubated at 37°C and 5% CO_2 for 24 h in medium alone or in presence of 1 µg/ml fludarabine (Sigma-Aldrich, MO, USA), 1 µg/ml mafosfamide (IIT GmbH, Bielefeld, Germany), or a combination of both. PBMCs were then washed and cultured for up to 6 days in complete medium.

Flow cytometry analysis

For characterization of the T cell pool, fresh blood samples or purified PBMCs were incubated for 15 min with a mixture of fluorochrome-labelled murine anti-human antibodies directed to the following antigens: CD19 (R-phycoerythrin, RPE), CD25 [fluorescein isothiocyanate (FITC)] (all from BD Biosciences, CA, USA), CD3 (phycoerythrin-cyanin 5, PC5),

CD4 [(energy-coupled dye (ECD)], CD4 (phycoerythrin-cyanin 7, PC7), CD8 (PC7), CD19 (PC7), CD38 (PE), CD45RA (FITC), CD45RA (ECD), CD62L (PE), CD127 (PE) (all from Beckman Coulter, CA, USA), CD4 (cyanin 5, Cy5), CD8 (FITC), CD8 (PC5) (all from DAKO, Glostrup, Denmark). Viability was assessed by staining cells with Annexin-V (FITC) (Alexis, Lausen, Switzerland) and 7-AAD (Beckman Coulter). Subsequently, samples were washed with PBS and analyzed immediately using the FC500 flow cytometer and the CXP1.0 software (Beckman Coulter).

For characterization of murine T cells, tail vein blood was stained with fluorochrome-labelled rat anti-mouse antibodies directed to the following antigens: CD3 (FITC), CD25 (PE), CD44 (cyanin 5.5), CCR4 (PC7), CXCR3 (Allophycocyanin; APC), CD4 (Pacific Blue) (all from BioLegend Europe, Uithoorn, the Netherlands) and CD8 (APC-H7) (BD Biosciences) for 15 min. Erythrocytes were lysed using BD FACS Lysing solution (BD Biosciences). Cells were then resuspended in PBS and analysed immediately using the Gallios flow cytometer (Beckman Coulter).

Allogeneic dendritic cell preparation and mixed lymphocyte reaction

Peripheral blood mononuclear cells were purified by density centrifugation from buffy coats from healthy donors retrieved from the local blood bank. Monocytes were enriched by CD14 MicroBead-positive selection (Miltenyi, Bergisch Gladbach, Germany) and cultivated in complete medium in the presence of recombinant human interleukin 4 (rhIL-4,500 U/ml) and granulocyte macrophage colony-stimulating factor (rhGM-CSF, 1,000 U/ml) (both from CellGenix, Freiburg, Germany) for 6 days. Fresh rhIL-4 and rhGM-CSF was added on day 3. On day 6, purity of differentiated dendritic cells was assessed by light microscopy and by flow cytometry (CD14-Cy5, CD11c-PE, CD83-FITC; Beckman Coulter). Cultures containing >90% dendritic cells, displaying high expression of CD11c and low expression of CD14 and CD83, were irradiated with 30 Gy and stored at −80°C until used.

Mixed lymphocyte reaction (MLR) was performed using CLL PBMCs as responders and allogeneic dendritic cells from six different buffy coats as stimulators. In brief, PBMCs from CLL patients were incubated for 24 h with fludarabine (1 μg/ml) or medium alone. The cells were then washed with PBS. Irradiated allogeneic dendritic cells were thawed and viability was assessed by trypan blue inclusion. CLL PBMCs and allogeneic dendritic cells were incubated with 5 μM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma, MO, USA) at a concentration of 10^6 cells/ml in PBS for 5 min. CFSE incorporation was stopped by adding complete medium and the cells were washed once with complete medium. PBMCs from CLL

patients were seeded into 24-well flat-bottom cell culture plates (Corning, MA, USA) at a concentration of 2.5×10^6 cells/ml/well. Irradiated CFSE-stained allogeneic dendritic cells were added at a stimulator/responder ratio of 1:10 and the coculture left untreated for 6 days. Using flow cytometry the extent of proliferation was determined at the end of the coculture period by assessing the fraction of cells which had lost incorporated CFSE upon cell division and therefore displayed reduced CFSE fluorescence intensity. In some experiments, instead of allogeneic dendritic cells polystyrene beads coated with a mixture of monoclonal antibodies against CD3 and CD28 (Dynabeads® Human CD3/CD28 T Cell Expander, Invitrogen, CA, USA) were added to the 6-day PBMC culture in a PBMC/bead ratio of 250:1.

Cytokine release measurement by cytometric bead array

Peripheral blood mononuclear cells from CLL patients were incubated for 24 h with fludarabine (1 μg/ml), mafosfamide (the active form of cyclophosphamide 1 μg/ml) or a combination of both. The cells were then washed, 5×10^6 PBMC were incubated either with medium alone or stimulated with phorbol 12-myristate 13-acetate (PMA 1 ng/ml) and ionomycin (0.5 μg/ml) (Sigma) for 6 days. After the stimulation period, the culture supernatants were collected and either immediately analyzed for presence of cytokines or stored at −20°C for later analysis.

For evaluation of T_H1 and T_H2 cytokine production after stimulation, we performed cytokine bead assays (CBAs) according to the manufacturer's recommendations (CBA Human T_H1/T_H2 Cytokine Kit II; BD Biosciences).

Murine tcl1tg tumour transplantation model

A tcl1a transgenic (tcl1tg) mouse, which has the coding region of human *tcl1a* placed under the control of a B cell-specific IgVH-promoter and IgH-Eμ enhancer was previously published as a mouse model for CLL. It develops clonal CD5-positive B cell leukaemia manifested by peripheral blood lymphocytosis and splenomegaly at a median of 11 months [25–27]. These mice were backcrossed onto pure C57BL/6 background to allow transfer of primary tcl1tg tumour cells into immune competent (not irradiated) syngeneic wild type C57BL/6 recipient mice. In the transplanted mice, the time to disease onset is shortened from 1 year to about 3 months, which vastly facilitates analysis (Hofbauer JP, Egle A, unpublished [26]). Tumour transplanted mice were treated with fludarabine (intraperitoneal injections of 35 mg/kg/day of fludarabine on 5 consecutive days) at the onset of disease (tumour load in peripheral blood >30%). Mice were bled from the tail vein at the indicated time points and T cell subsets were

analysed. CD4^{+} and CD8^{+} memory cells were defined by low expression of CD25 and high expression of CD44. T_H1 and T_H2 cells were defined by expression of the chemokine receptors CXCR3 (T_H1) and CCR4 (T_H2).

Statistical analysis

All statistical analyses were performed using PASW Statistics 17 (SPSS Inc. IL, USA) or Graph Pad Prism 5. Boxplots show median (horizontal line in box), difference between 25th and 75th percentile (length of box) and data range (whiskers) unless stated otherwise. Outliers are marked by circles. Values in the results section are in general mean ± standard deviation and p value (paired or unpaired student's t test). Graphics were created using PASW Statistics 17 and Graph Pad Prism 5.

Results

Characterization of T cells after cytotoxic treatment

Peripheral blood mononuclear cells from previously untreated CLL patients were isolated as described in "Materials and methods". For all in vitro experiments, we used mafosfamide, the active form of cyclophosphamide, since cyclophosphamide requires in vivo processing in the liver. Cells were incubated in medium containing clinically achieved concentrations of fludarabine (Flu 1 µg/ml), mafosfamide (Maf 1 µg/ml), or a combination of both agents (FluMaf) [28]. After 24 h, cells were washed, counted and cultivated for an additional period of 6 days. Viability of CD4^{+} and CD8^{+} T cells and of CD19^{+} CLL cells was determined on day 0 and day 6 by the Annexin V binding assay (Table 2). While there was no significant reduction in viability detectable immediately after the 24-h incubation with any of the single agents, the combined

FluMaf treatment significantly reduced viability of tumour cells and T cells when compared with medium control. Six days post-treatment PBMCs showed significantly reduced viability in the CD4^{+}, CD8^{+} T cell as well as in the CD19^{+} CLL cell compartment. We found no significant correlation of any of the investigated clinical risk parameters (Table 1) with in vitro sensitivity of T cells to fludarabine or mafosfamide. Additionally, we calculated the ratio between CLL cells and T cells before and 6 days after cytotoxic treatment. We found a significant increase in the CLL/T cell ratio (Table 2) indicating a higher sensitivity of T cells to cell death induced by fludarabine and mafosfamide when compared with CLL cells.

At the end of the 6-day culture period, T cells surviving cytotoxic treatment were characterized with respect to expression of the activation marker CD38, the relative distribution of naïve and memory T cells and the relative proportion of Tregs within the T cell pool. The difference in the numbers of the patient samples in Fig. 1 is based on the strong reduction in cell numbers following cytotoxic treatment in some cases that did not allow analysis. However, all statistical analyses were calculated from paired samples. We found a significant reduction in the percentage of CD38-expressing CD4^{+} T cells after treatment with mafosfamide (26.6% ± 12.8, $p = .001$) and FluMaf (22.2% ± 20.3, $p = .048$) when compared with untreated PBMCs (38.3% ± 20.4), while fludarabine treatment had no effect on CD38 expression (35.6% ± 24.4, $p = .063$) (Fig. 1a). In the CD8^{+} T cell pool, such a reduction of relative numbers of CD38^{+} T cells after treatment with mafosfamide or FluMaf was also observed, yet to a lower extent probably due to the generally lower CD38 expression on CD8^{+} T cells (Fig. 1b; control 16.6% ± 9.7; Flu 20.2% ± 12.2, $p = .461$; Maf 12.5% ± 5.0, $p = .031$; FluMaf 3.6% ± 3.1, $p = .112$). When we compared the effect of cytotoxic treatment on absolute cell numbers, we found that CD38^{+} T cells were more sensitive to mafosfamide-induced

Table 2 Viability (%) and CLL/T cell ratio after 24 h cytotoxic treatment (Day 0) or subsequent 6 day culture in medium (Day 6)

	CD4^{+} T cells	CD8^{+} T cells	CLL cells	CLL/T cell ratio
Day 0				
Medium	91.2% ± 1.8	87.8% ± 2.8	68.9% ± 5.0	20.7 ± 4.2
Flu	89.6% ± 1.9; $p = .204$	80.1% ± 3.5; $p = .135$	65.2% ± 5.9; $p = .421$	24.7 ± 25.5; $p = .155$
Maf	91.9% ± 1.7; $p = .462$	85.2% ± 3.8; $p = .284$	68.5% ± 6.7; $p = .591$	19.1 ± 4.5; $p = .435$
FluMaf	**88.2% ± 2.0; $p = .024$**	**73.3% ± 3.6; $p < .001$**	**45.4% ± 6.6; $p = .022$**	22.4 ± 7.4; $p = .432$
Day 6				
Medium	86.3% ± 2.6	80.1% ± 4.2	61.0% ± 6.8	15.9 ± 2.9
Flu	**40.3% ± 5.6; $p < .001$**	**26.6% ± 5.7; $p < .001$**	**35.1% ± 6.6; $p = .001$**	**43.9 ± 9.9; $p = .004$**
Maf	**63.4% ± 6.1; $p < .001$**	**50.9% ± 6.6; $p < .001$**	**47.6% ± 9.8; $p = .015$**	**23.2 ± 5.7; $p = .027$**
FluMaf	**15.8% ± 4.7; $p < .001$**	**6.4% ± 2.7; $p < .001$**	**5.7% ± 7.2; $p < .001$**	**65.6 ± 16.4; $p = .001$**

P values reflect treatment compared to untreated medium control. Significant differences are highlighted in bold ($n = 16$)

Fig. 1 PBMCs from 27 untreated CLL patients were incubated for 24 h with complete medium alone, fludarabine (1 μg/ml), mafosfamide (1 μg/ml) or both. The cells were then washed and cultured for subsequent 6 days in complete medium. On day 6, the fraction of CD38-expressing CD4$^+$ (**a**) or CD8$^+$ (**b**) T cells was determined. T cell subsets were defined as stated in the Results section and the fraction of memory CD4$^+$ (**c**) and CD8$^+$ (**d**) T cells was determined. Values significantly different from untreated controls are indicated by *asterisks* (*$p < .05$; **$p < .01$; ***$p < .001$)
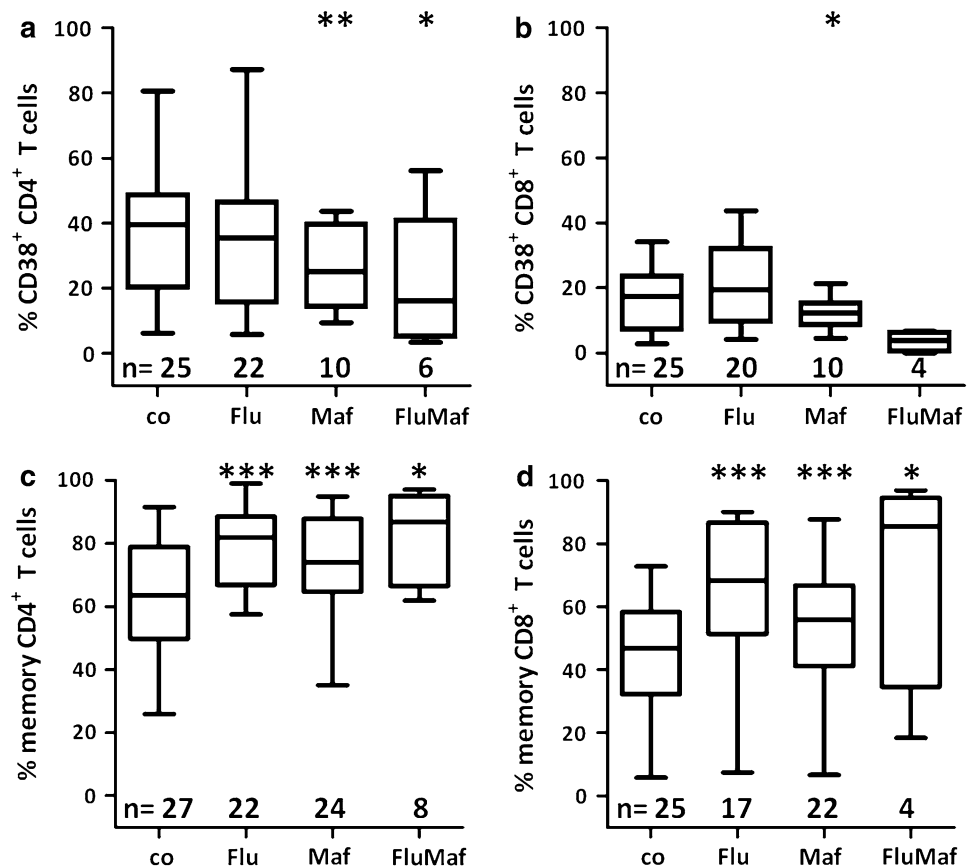
apoptosis than T cells lacking CD38 expression (not shown). None of the clinical risk parameters depicted in Table 1 correlated with altered CD38 expression on T cells after cytotoxic treatment.

Tregs were defined by high CD25 (IL-2Rα) and low CD127 (IL-7Rα) expression levels within the CD4$^+$ T cell compartment. We found no significant changes in the percentage of Tregs after treatment with any of the cytotoxic agents alone or in combination (data not shown).

The expression profile of the leucocyte homing receptor L-selectin (CD62L) and CD45RA was used to distinguish between naïve (CD45RA$^+$, CD62L$^+$) and memory (CD45RA$^-$) T cells (Fig. 1c, d). When compared to untreated controls, fludarabine- or mafosfamide-treated CD4$^+$ T cells showed an elevated memory subset fraction (63.3% ± 16.4 vs. 79.3% ± 13.0, $p < .001$, or 73.1% ± 15.8, $p < .001$, respectively) that further increased following combined treatment with the two agents (82.4% ± 14.0, $p = .016$). Correspondingly, the percentage of naïve cells was decreased. In the CD8$^+$ T cell compartment, a shift towards a memory phenotype could also be observed after cytotoxic treatment (control 45.6% ± 17.1; Flu 66.4% ± 23.2, $p < .001$; Maf 53.8% ± 20.0, $p < .001$; FluMaf 71.5% ± 35.9, $p = .021$).

Additionally, we evaluated the effect of fludarabine and mafosfamide on T cells from healthy donors. The effects observed in CLL T cells were recapitulated in the healthy T lymphocytes suggesting that these alterations are not disease-specific (Suppl. Fig. 2a–d).

Functionality of in vitro fludarabine-treated T cells: proliferation

To evaluate the ability of CLL T cells to proliferate following cytotoxic treatment, we performed MLR. PBMCs from 16 untreated CLL patients were incubated for 24 h with fludarabine or medium alone. The cells were then washed and co-cultured with allogeneic dendritic cells (DC) as stimulators and after 6 days T cell proliferation rates were measured by the CFSE dilution assay.

Upon stimulation with DC, the proliferating fraction of fludarabine-treated CD4$^+$ and CD8$^+$ T cells was significantly higher than that of untreated T cells (43.4% ± 5.0 vs. 25.7% ± 5.1, $p < .001$ and 40.9% ± 6.7 vs. 23.1% ± 6.0, $p < .001$, respectively) (Fig. 2a). To exclude the possibility that increased proliferation was just a phenomenon of increased stimulator:responder ratios due to the massive cytoreduction by fludarabine treatment, we correlated the proliferation rates after 6 days with the extent of cytoreduction by fludarabine. We could not observe any correlation between cell reduction by fludarabine treatment and proliferation induction by DC (not shown, Pearson correlation:
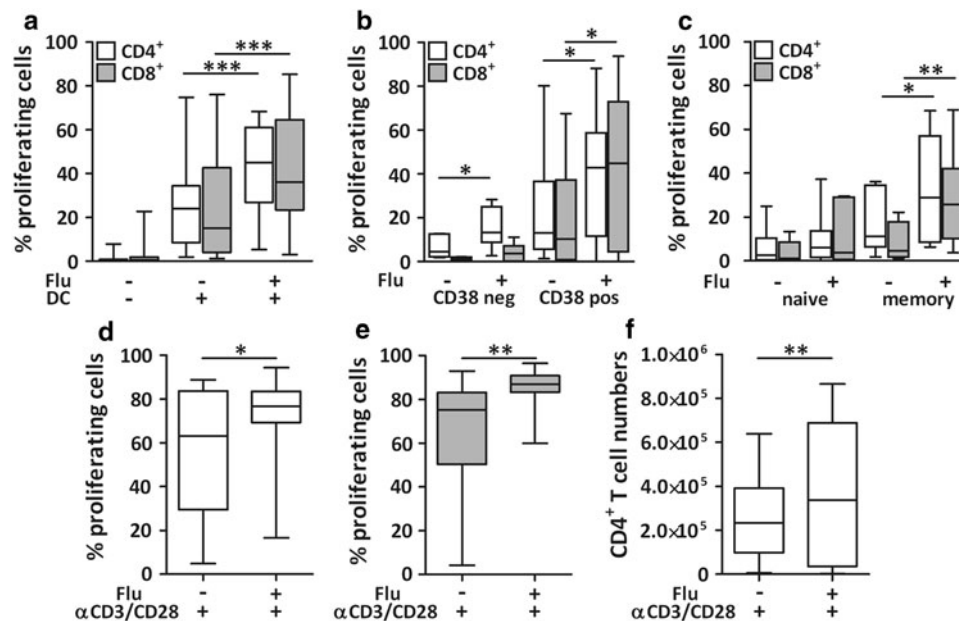
Fig. 2 **a** PBMCs from CLL patients ($n = 16$) were incubated for 24 h with medium alone, or fludarabine (1 µg/ml). The cells were then washed, stained with CFSE, and cultured for 6 days in the presence of allogeneic DC at a ratio of 10:1. On day 6, the fraction of CFSE-diluted T cells was assessed. The fraction of proliferating T cells in CD38-positive or negative T cell population (**b**) and in naïve or memory T cell compartment (**c**) was measured ($n = 8$). **d** PBMCs from CLL patients ($n = 16$) were incubated for 24 h with medium alone, or fludarabine (1 µg/ml). The cells were then washed, stained with CFSE, and cultured for 6 days in the presence of beads coated with anti-CD3/anti-CD28 antibodies at a PBMC/bead ratio of 250:1. On day 6, the fraction of CFSE-diluted CD4$^+$ (**d**) and CD8$^+$ (**e**) T cells and the total CD4$^+$ T cell number (**f**) were assessed. Significance is indicated by asterisks (*$p < .05$, **$p < .01$, ***$p < .001$) (*white bars* CD4$^+$, *grey bars* CD8$^+$ T cells)

0.216, $p = .389$), ruling out a bias generated by variable stimulator:responder ratios.

We next assessed the phenotype of the T cells expanding in response to DC stimulation and therefore characterized expression levels of CD38, CD62L and CD45RA on CD4$^+$ and CD8$^+$ T cells on day 6 of the MLR. CD38-expressing T cells in general showed a higher proliferative activity than T cells not expressing CD38 (Fig. 2b). When PBMCs had been treated with fludarabine, increased proliferation was detected both in the CD4$^+$CD38$^+$ (26.5% ± 10.1 vs. 46.2% ± 10.0, $p = .024$) and in the CD4$^+$CD38$^-$ T cell pool (7.3% ± 1.9 vs. 16.0% ± 3.6, $p = .031$), whereas the increase in proliferation after fludarabine treatment was significant in the CD8$^+$CD38$^+$ population (21.4% ± 9.5 vs. 49.2% ± 12.8, $p = .011$) and borderline significant in the CD8$^+$CD38$^-$ T cell pool (1.3% ± 0.3 vs. 4.7% ± 1.5, $p = .054$). We also determined whether there was a difference in the capacity of naïve and memory T cells to proliferate upon allogeneic stimulus after fludarabine treatment. We observed a significantly higher proportion of proliferating T cells in the memory compartment as compared to naïve T cells. Interestingly, naïve T cells showed no difference in proliferation after fludarabine treatment, whereas both CD4$^+$ and CD8$^+$ memory T cells, when pre-treated with fludarabine, showed higher proliferation than the respective untreated controls (Fig. 2c; CD4$^+$: 16.0% ± 5.2

vs. 34.0% ± 9.9, $p = .030$; CD8$^+$: 8.3% ± 3.1 vs. 29.7% ± 8.3, $p = .009$). In order to confirm these results in a setting that is independent of allogeneic antigen recognition, we performed proliferation assays using beads coupled with αCD3 and αCD28 antibodies instead of allogeneic DC. The CD4$^+$ and CD8$^+$ T cell proliferation rate upon CD3/CD28 bead stimulation was significantly higher when the cells were pretreated with fludarabine for 24 h (Fig. 2d, e; CD4$^+$: 56.7% ± 29.1 vs. 74.5% ± 17.8, $p = .021$; CD8$^+$: 63.1% ± 27.7 vs. 84.9% ± 9.4, $p = .005$). Additionally, absolute CD4$^+$ T cells numbers after CD3/CD28 bead stimulation were significantly increased in the fludarabine-pretreated samples ($p = .004$), thus confirming and underlining the positive influence of fludarabine on T cell proliferation in an antigen-independent setting (Fig. 2f). Fludarabine-induced increase in T cell proliferation after CD3/CD28 beads was recapitulated in healthy T lymphocytes (Suppl. Fig. 2e, f).

Functionality of in vitro fludarabine-treated T cells: cytokine secretion

T$_H$1-type cytokines such as interferon gamma (IFNγ), interleukin 2 (IL-2) and tumour necrosis factor alpha (TNFα) are considered key cytokines for T cell-mediated tumour control, while T$_H$2 cytokines, as IL-10, IL-4 and IL-6,

counteract differentiation of T cells to a T_H1 subtype. We therefore investigated the ability of fludarabine- or mafosfamide-treated T cells to produce T_H1 or T_H2 cytokines upon stimulation with PMA/ionomycin.

We incubated PBMCs from untreated CLL patients with fludarabine (1 μg/ml), mafosfamide (1 μg/ml) or DMSO (medium control) for 24 h. The cells were then washed and were left untreated or stimulated for additional 6 days with PMA (1 ng/ml) and ionomycin (0.5 μg/ml). We collected the culture supernatants and evaluated the concentration of secreted cytokines by CBA.

As depicted in Fig. 3, we found that after 6 days of stimulation with PMA/ionomycin, the supernatant of PBMCs pre-treated with fludarabine showed significantly elevated levels of the T_H1 cytokines IFNγ ($p = .025$) and IL-2 ($p = .024$). Mafosfamide did not significantly alter the levels of the two T_H1 cytokines IFNγ ($p = .065$) and IL-2 ($p = .658$). Furthermore, the T_H2 cytokine IL-10 was significantly decreased in fludarabine- ($p = .016$) but not in mafosfamide- ($p = .219$) treated samples (Fig. 3). While TNFα levels remained nearly unaffected when compared to untreated controls, IL-4 and IL-6 levels were either below the detection limit of the CBA or, in the samples where detection was possible, not significantly altered by either fludarabine or mafosfamide treatment (data not shown). To ascertain that the elevated T_H1 cytokine levels originate from T cells and not from the CLL cells, we performed intracellular IFNγ staining of PBMCs after 24 h fludarabine treatment followed by 4 h PMA/ionomycin stimulation. We did not detect intracellular IFNγ in $CD19^+$ $CD5^+$ CLL cells, but IFNγ-positive cells within the $CD19^-$ $CD5^+$ T cell population (Suppl. Fig. 1).

Characterization of CLL T cells after in vivo fludarabine/cyclophosphamide (FC) treatment

Since we observed changes in the T cell compartment after in vitro treatment of CLL PBMCs with fludarabine or

mafosfamide with respect to activation status, differentiation, proliferation and cytokine secretion, we finally tested whether treatment in vivo would yield similar results. We therefore analyzed samples from chemonaïve CLL patients who started a modified FC therapy protocol at our clinical department. This protocol includes administration of fludarabine (25 mg/m²/day i.v.) and cyclophosphamide (250 mg/m²/day i.v.) over 4 days. We used samples drawn before therapy (d0) and on day 1, 2 and 3 of the first cycle for expression and functional studies.

For functional analysis of T cell proliferation we collected blood samples from FC-treated patients before treatment, purified the lymphocytes and incubated them in vitro for 24 h with or without 1 μg/ml fludarabine. In addition, PBMCs from samples drawn after 1 or 3 days of in vivo FC treatment were isolated. Cells were washed, stained with CFSE and co-cultured for subsequent 6 days with DC at a ratio 10:1. For each sample, the percentage of proliferating T cells was measured on day 6 of the co-culture period (Fig. 4a). When compared with untreated control cells, in vitro incubation with fludarabine increased the percentage of proliferating $CD4^+$ (15.9% ± 5.3 vs. 24.2% ± 8.2, $p = .049$) and $CD8^+$ (21.2% ± 4.0 vs. 58.6% ± 3.8, $p = .001$) T cells. While there was no increase in T cell proliferation after 24 h in vivo FC treatment, we detected increased fractions of proliferating T cells after 3 days in vivo FC treatment ($CD4^+$ 15.9% ± 5.3 vs. 33.1% ± 8.7, $p = .010$; $CD8^+$ 21.2% ± 4.0 vs. 75.7% ± 8.4, $p = .003$).

We also compared expression of CD38 on CLL T cells on day 0 and day 3 of the therapy and observed a slight increase in $CD4^+CD38^+$ (54.3% ± 2.8 vs. 58.3% ± 3.8, $p = .387$) and $CD8^+CD38^+$ T cells (51.1% ± 4.2 vs. 59.4 ± 4.9, $p = .210$) (Fig. 4b), that was not significant. The fraction of Tregs within the $CD4^+$ T cells remained stable over the first 3 days of therapy (not shown). We finally evaluated any changes in T cell subset distribution between day 0 and day 3 of the FC treatment. The memory fraction remained stable or even slightly decreased during this
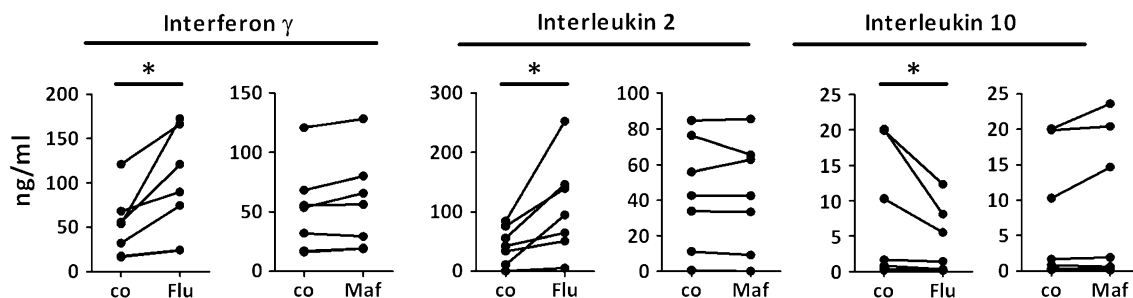
Fig. 3 PBMCs from seven untreated CLL patients were incubated with medium alone (*co*), fludarabine (Flu 1 μg/ml) or mafosfamide (Maf 1 μg/ml) for 24 h. The cells were then washed and incubated for further 6 days in presence of PMA (1 ng/ml) and ionomycin (0.5 μg/ml) at a concentration of 2.5×10^6 cells/ml. Culture supernatants were collected and the concentration of cytokines (ng/ml) was measured by cytokine bead assay (*CBA*). Values significantly different from untreated controls are indicated by *asterisks* (**p* < .05)

Fig. 4 a Chemonaïve CLL patients were treated with fludarabine and cyclophosphamide. PBMCs collected before treatment were incubated for 24 h in presence (vitro24) or absence of 1 μg/ml fludarabine. PBMCs were also collected on day 1 (vivo24) and day 3 (vivo72) of the FC treatment. All samples were co-cultured in vitro for 6 days with allogeneic DC and the T cell proliferation rates on day 6 were measured by CFSE dilution. *Boxplots* indicate the percentage of proliferating CD4$^+$ (*white bars*) or CD8$^+$ (*grey bars*) T cells (n = 6). **b–d** Peripheral blood was collected before treatment (0) and after 24 or 72 h of therapy (n = 16). Whole blood staining was performed of each sample and the percentage of (**b**) CD38$^+$ T cells as well as the T cell subset distribution of (**c**) CD4$^+$ and (**d**) CD8$^+$ T cells were determined by flow cytometry (naïve T cells in normal frames, memory T cells in bold frames)
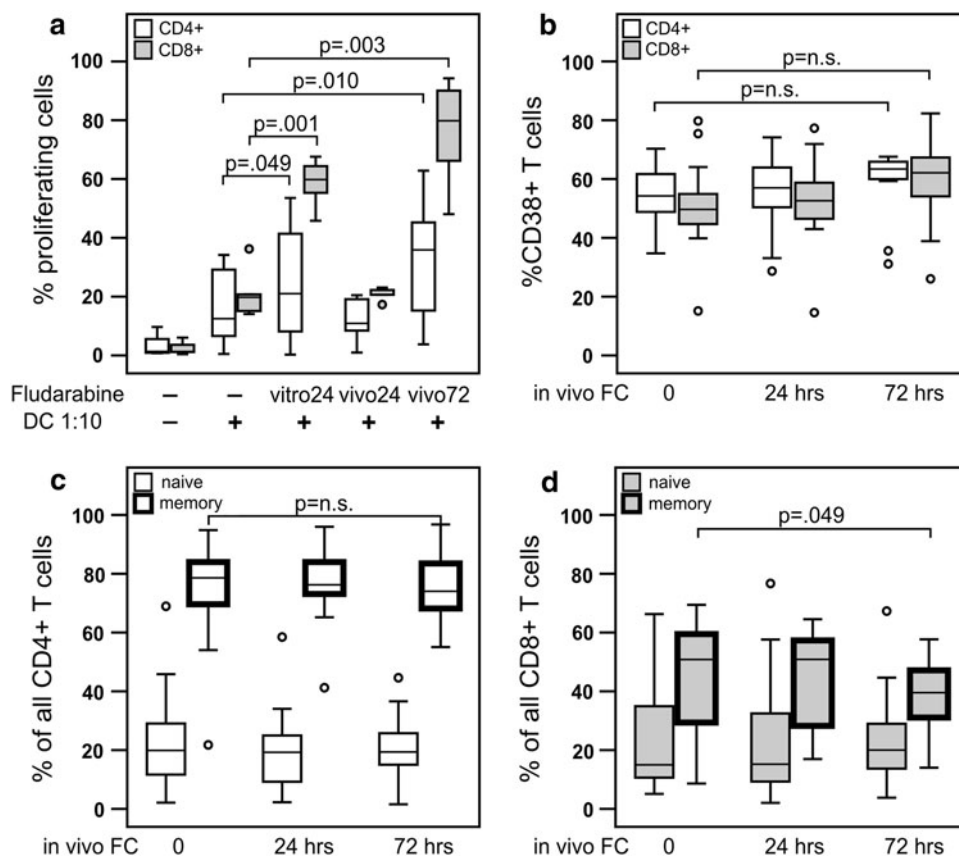
period in the CD4$^+$ (Fig. 4c, 74.3% ± 4.4 vs. 60.9% ± 7.9, p = .150) and in the CD8$^+$ (Fig. 4d, 45.3% ± 4.5 vs. 31.6% ± 4.9, p = .049) T cell compartment.

Characterization of T cells after in vivo fludarabine treatment of tcl1 transgenic tumour transplanted mice

Since treatment of our patient cohort included administration of rituximab [29] on day 4 of therapy, possible long-term alterations in the T cell compartment following fludarabine/cyclophosphamide treatment in vivo could not be assessed in these patients. Therefore, we used tcl1tg mice as a CLL mouse model to address this question. We transplanted primary tcl1tg tumour cells into syngeneic wildtype mice. At the onset of disease, we treated these mice with fludarabine and monitored changes in the peripheral T cell subsets. As shown in Fig. 5, we observed an increase in the percentage of cells with a memory phenotype within the CD4$^+$ and the CD8$^+$ T cell compartment (memory CD4$^+$: d-3, 42.9 ± 13.7; d3, 70.0 ± 14.6, p < .001; d7, 74.3 ± 15.6, p < .001; d11, 60.2 ± 33.9, p = .338; memory CD8$^+$: d-3, 57.6 ± 13.2; d3, 87.2 ± 5.2, p = .002; d7, 80.2 ± 6.3, p = .002; d11, 81.5 ± 9.9, p < .001) In addition and in line with the results from our in vitro study of the human model, the absolute numbers of T$_H$1 cells increased during treatment while T$_H$2 cell numbers declined (Fig. 5c).

Discussion

Chemoimmunotherapy of lymphatic malignancies aims at the reduction of tumour load on the one hand and establishment of long-lasting tumour control by the patient's immune system on the other. Studies in patients with refractory metastatic melanoma have recently shown that lymphodepletion, induced by fludarabine/cyclophosphamide treatment, is associated with increased serum levels of the lymphocyte homeostatic cytokines IL-7 and IL-15, and that subsequent adoptive cell therapy can mediate significant tumour regression [30, 31]. Here, we show that cytotoxic treatment with fludarabine despite its negative impact on lymphocyte numbers in vitro and in vivo positively interacts with T cell functions, and that some of these effects synergize with cyclophosphamide, a combination used as backbone of modern CLL treatment.

We observed that CD4$^+$ CD38$^+$ T cells exhibited a higher sensitivity to in vitro treatment with fludarabine/mafosfamide than the CD38$^-$ T cell counterpart, resulting in a decrease in percentage of CD38$^+$ CD4$^+$ T cells. CD38 expression on T cells has been shown to reflect chronic activation and stimulation in various diseases [32, 33] and also in acute graft rejection [34]. Our own group has recently shown that the levels of CD38 expression on T cells predict the course of the disease in male CLL patients
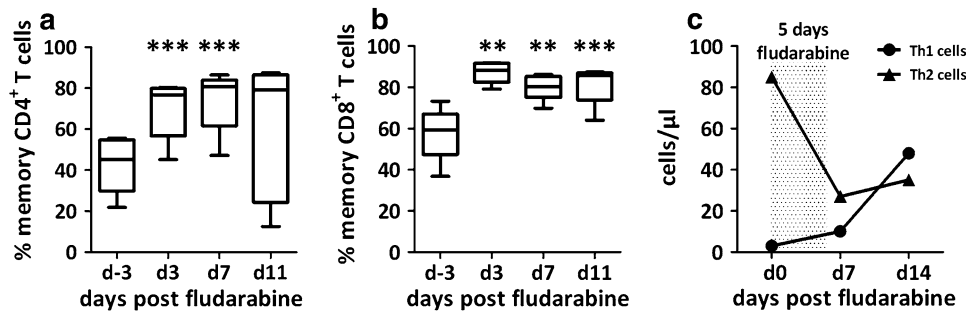
Fig. 5 **a** Peripheral blood from five mice was collected 3 days prior to (d-3), during (d3) and after (d7, d11) fludarabine treatment (5 days; 35 mg/kg/day). T cells were stained using specific antibodies and the percentage of CD4$^+$ (**a**) and CD8$^+$ (**b**) memory T cells was assessed. **c** In a separate experiment, the numbers of peripheral blood T$_H$1 and T$_H$2 CD4$^+$ T cells prior to (d0) and after fludarabine treatment (d7, d14) were determined. One representative result of two experiments is shown

with higher CD38 expression correlating with a worse clinical prognosis [35], and this was confirmed by the recent study by Abousamra et al. [36]. According to these data, CD38$^+$ T cells, despite their activated phenotype, seem to fail to perform sufficient tumour control in CLL but rather support maintenance or even expansion of the malignant clone. Hence, preferential depletion of CD38$^+$ T cells during FC treatment as observed in this study in vitro could well add to the therapeutic efficacy of this drug combination in CLL.

Furthermore, we observed that CD4$^+$ and CD8$^+$ memory T cells were more resistant than naïve T cells to fludarabine and mafosfamide treatment in vitro leading to a decrease in the naïve/memory T cell ratio. These results are in line with data from Koike et al. [37] who have shown in a melanoma mouse model that adoptively transferred naïve T cells displayed an activated memory phenotype after lymphodepletion with cyclophosphamide and fludarabine. Additionally, in psoriatic arthritis and multiple sclerosis, increased resistance of memory T cells towards fludarabine or cyclophosphamide has been shown [38, 39]. Interestingly, when we stimulated CLL PBMCs after fludarabine treatment with DC or CD3/CD28 beads, we observed an increase in T cell proliferation as compared to untreated control PBMCs. The increase in proliferation was mainly found in the memory T cell subpopulations and was only observed after fludarabine treatment, while mafosfamide, although inducing similar levels of apoptosis, had no effect on proliferation rates (data not shown). Since cytotoxic therapy not only reduces the tumour load but also a significant proportion of immune cells involved in tumour control, rapid reconstitution of the T cell compartment after therapy seems crucial in order to reduce the chance for cancer relapse. Fludarabine, by enhancing the proliferative capacity especially of memory T cells, might thereby contribute positively to long-lasting tumour control.

Gamberale et al. [40] have shown that IFNγ production upon PMA/ionomycin stimulation of CD2$^+$ T and NK cells

from CLL patient samples or healthy donor lymphocytes was significantly increased after 24 h when fludarabine was added to the culture. Additionally, they showed that fludarabine did not reduce STAT1 expression, which is activated in response to IFNγ, in PBMCs or CD2$^+$ cells from CLL patients or healthy donors, contrary to what had been reported before [41]. When we tested T cell functionality from CLL patients we also found that fludarabine treatment enhances the capacity of PBMCs to produce IFNγ upon PMA/ionomycin stimulation. Yet, we only observed a significant increase of secreted IFNγ after a 6 days post-treatment culture. This is probably due to the lower percentage of T cells (8.4% ± 2.4) in our analysed CLL samples when compared to the samples analysed by Gamberale et al. [40] (at least 15% CD2+ cells). In extend to that, we could show that in vitro treatment with fludarabine, but not mafosfamide enhanced the production of the T$_H$1 cytokine IL-2 as well, whereas the T$_H$2 cytokine IL-10 was decreased, indicating that in our experimental setting fludarabine promoted T$_H$1 differentiation during T cell activation.

Notably, the observed T cell alterations occurred irrespective of the initial CLL/T cell ratio and were also recapitulated in T cells from healthy donors.

In contrast to what we found in our in vitro model, no changes in the ratios of memory/naïve or regulatory T cells could be observed in patients in vivo and also the fraction of CD38$^+$ T cells was stable. Protection from apoptosis provided by the micro-environment in vivo might be responsible for this discrepancy. Alternatively, treatment for a longer period would be necessary to reveal the modulating effect of fludarabine on T cell subsets in vivo.

The results from the tcl1tg mouse model in which we could follow possible T cell changes for up to 14 days after in vivo fludarabine treatment corroborate our results from the in vitro model and thereby support the time factor as one possible explanation. Also, when we stimulated PBMCs from CLL patients treated with fludarabine and cyclophosphamide for 3 days with DC, we again observed

a significant increase in T cell proliferation when compared with non-treated samples. This indicates that FC treatment lowers the threshold for subsequent T cell expansion by other means than selective cytodepletion.

In conclusion, our results provide new evidence that fludarabine is not only cytotoxic but also able to modulate distinct functions of the immune system, in particular to stimulate a shift from naïve to memory T cell subsets, to lower the threshold for proliferation and to increase T_H1 cytokine production. Hence, besides reducing the tumour load, fludarabine-induced immune modulation may on the one hand improve post-treatment tumour control, but on the other hand also account for the observation that fludarabine mono- or combinational therapy increases the incidence of auto-immune reactions, predominantly autoimmune haemolytic anaemia [42, 43].

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