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## Research Article

# T Regulatory Cells in Relapsed/Refractory Chronic Lymphocytic Leukemia Treated with High Dose Methylprednisolone and Rituximab

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### ABSTRACT

Higher circulating T regulatory cell (Treg) numbers have been found in untreated patients with chronic lymphocytic leukemia (CLL) compared to healthy subjects and correlated with progressive disease as well as time to first treatment in low-risk patients [1]. Some agents can reduce Treg numbers in CLL patients, but there are no data on the prognostic role of Treg dynamics and patient outcome. We present data from the LT-CLL-001 study, in which the clinical benefit of dose-dense high dose methylprednisolone (HDMP) and rituximab (Rtx) combination in relapsed or refractory high-risk patients with CLL was evaluated [2]. During the study, the change of T regulatory cell frequencies was measured in relation to overall response rate (ORR), progression-free survival (PFS), and overall survival (OS). Twenty-nine CLL patients with clinically or biologically high-risk disease were included. Treg frequency was evaluated at screening, after three treatment courses, and at the end of therapy. Significant reduction of the median frequencies of Treg during treatment was observed: median (range) of Treg<sup>0-3</sup> after three treatment courses was 2.14% (-1.84%-9.42%),  $p < 0.001$  and median (range) of Treg<sup>0-6</sup> was 1.01% (-2.95%-8.35%,  $p = 0.004$ ). Patients with deeper Treg reduction between screening and three treatment courses had significantly better PFS and OS (Table 1 & 2). Our data for the first time show that HDMP and Rtx combination reduces Treg frequency in pretreated CLL patients. Early and deeper Treg reduction is an independent prognostic factor for longer PFS and OS. (ClinicalTrials.gov identifier: NCT005 58181).

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## Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia among adults in western countries [3]. The significant heterogeneity in the course of the disease is reported and a number of prognostic parameters like mutation status of immunoglobulin variable heavy chain (*IgVH*) genes or the presence of genetic abnormalities may predict the prognosis and distinguish those who could develop aggressive disease and need immediate treatment [4]. Among prognostic factors, the del 17p or mutation of the *TP53* gene are associated with a worse prognosis and resistance to immunochemotherapy [4-6].

CLL is known for significant dysregulation of the immune system, most typically immune suppression, already in the early stages, which deteriorates regardless of the disease progression [1, 7, 8]. Several authors reported data on T regulatory cells in CLL, showing in the majority of cases an expansion of this population. In addition, a correlation between higher Treg numbers and more aggressive clinical-biological features and adverse prognosis of CLL has been described [1, 7, 8]. To date, the prognostic value of Treg frequencies in response to therapy and survival has not been reported. In this study, we show that HDMP and Rtx combination in patients with high-risk CLL leads to early decrease in Treg frequencies, and deeper reduction is associated with longer progression-free and overall survival.

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## Patients and Methods

### I Study Design

Two-center, single-arm, open-label, prospective study was conducted to evaluate the efficacy of dose-dense HDMP in combination with Rtx in pretreated CLL patients with clinically or biologically high-risk disease. Inclusion criteria and treatment regimen were described previously [2]. During the course of the study, Treg frequencies were measured at predefined time points: at screening (Treg<sup>0</sup>), after three treatment courses (Treg<sup>3</sup>), and after treatment completion (Treg<sup>6</sup>). Treg frequencies and their change during the course of treatment were correlated with response, prognostic factors and survival of the patients.

The study protocol was approved by the Lithuanian Bioethics Committee, and the study was conducted according to the Declaration of Helsinki. All patients provided written informed consent.

### II T Regulatory Cell Evaluation

T regulatory cells, defined as CD4+CD25+CD127-FoxP3+ cells, were detected as the percentage of total CD4+ T lymphocytes and were assessed in peripheral blood at screening, after three treatment courses and two months after the last treatment course. Venous blood of the patients was collected under standard conditions into a Vacutainer tube with K3EDTA anticoagulant (BD, USA). Samples were stained with monoclonal antibodies for direct immunofluorescence testing. Monoclonal antibodies for 6 colour analysis from Becton Dickinson (USA), BD Pharmingen™, and eBioscience. conjugated with FITC fluorochrome (CD25, CD57, CD4), PE fluorochrome (CD127, CD16, CD3), PerCP-Cy5.5 fluorochrome (CD4, CD3, CD45), APC fluorochrome (Foxp3, mouse IgG1 isotype control κ, CD45, CD19), PE-Cy7 fluorochrome (CD56), and APC-Cy7 fluorochrome (CD8) were used. Samples were prepared in accordance with Lysed Whole Blood technique. 100 μl of blood was first incubated with the aforementioned monoclonal antibodies for cell surface staining. Then samples were treated with BD Lysing Solution and were washed twice. For intracellular marker detection, cell membranes were permeabilized with permeabilizing solution 2. Afterwards, samples were incubated with monoclonal antibodies against intracellular markers (Foxp3, mouse IgG1 isotype control κ). After the washing procedure, cells were resuspended in PBS solution. Samples were run on FACS Canto (BD, USA) flow cytometer; data collection and analysis were performed with FACSDiva software version 6.1.2.

### III Patients

Eligible patients were diagnosed with CD20+ CLL with treatment indications according to the National Cancer Institute Working Group (NCI-WG) 1996 guidelines [9]. Patients had relapsed or progressive disease after at least one prior chemotherapy regimen, and clinically or biologically high-risk CLL was defined as progressive or stable disease while on fludarabine treatment, or relapse after fludarabine treatment within 12 months, and/or the presence of at least one of the following genetic factors: 17p deletion, 11q deletion, or trisomy 12 confirmed by fluorescence *in situ* hybridization (FISH), or TP53 mutation confirmed by sequencing. All patients were older than 18 years.

## IV Statistical Methods

Statistical analysis of NCI-WG response and survival were performed on an intent-to-treat basis and included all enrolled patients. Response to treatment (overall response rate, ORR) was expressed as the proportion of patients who achieved at least partial response (PR). Progression-free survival (PFS) was defined as the interval from entry into the study to disease progression or death, with surviving patients censored at the last follow-up date. Overall survival (OS) was defined as the duration between the date of entry into the study and death. Treg difference between patient subgroups was evaluated by the Mann-Whitney U test. Wilcoxon signed rank test was used to compare Treg<sup>0</sup>, Treg<sup>3</sup> and Treg<sup>6</sup> time points distributions. Cox regression model was used to evaluate the impact of different prognostic factors on PFS and OS. Survival estimates were plotted by the Kaplan-Meier method. Log-rank test was used to evaluate the difference between two survival curves. Two-tailed P values <0.05 were considered significant. Statistical analysis was performed using Statistical Analysis System (SAS) package version 9.2.

## Results

### I Patient Characteristics

Between September 2007 and January 2009, 29 patients were enrolled in the study. Patient demographics, baseline characteristics, response to treatment, and survival data were reported previously [2]. Updated median follow-up for all the patients was 29 (0-137) months, the median OS was 31 months (18-36), 7-year and 10-year survival were 17.2% (6.3-32.7%) and 6.9% (1.2-19.8%), respectively. Only two patients are alive, both after allogeneic bone marrow transplantation.

### II Response to Treatment

Eighteen patients responded to treatment, resulting in an ORR of 62%, all patients obtained partial response [2]. Treg<sup>0</sup> frequency did not correlate with response to treatment ( $p = 0.911$ ) or with other prognostic factors such as bulky lymphadenopathy ( $p = 0.674$ ), fludarabine refractoriness ( $p = 0.449$ ) or adverse cytogenetics ( $p = 0.268$ ).

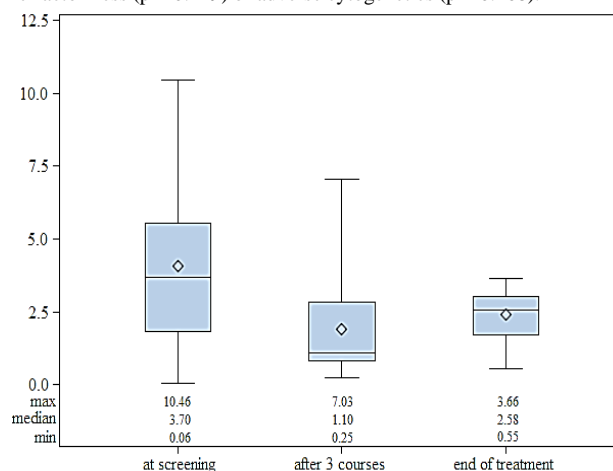


Figure 1: T regulatory cell frequency during treatment period.

### III The Change in T Regulatory Cell Frequency During Treatment

Treg were evaluated in 29 patients at Treg<sup>0</sup>, in 28 patients at Treg<sup>3</sup> and in 24 patients at Treg<sup>6</sup> time points. The median (range) Treg<sup>0</sup> frequency was 3.70% (0.06%-10.46%). There was a significant decrease in Treg frequency after three treatment courses (Treg<sup>0-3</sup>) and at the end of therapy (Treg<sup>0-6</sup>): median (range) of Treg<sup>0-3</sup> and Treg<sup>0-6</sup> was 2.14% (-1.84%-9.42%),  $p < 0.001$  and 1.01% (-2.95%- 8.35%,  $p = 0.004$ ). Slight progression of Treg<sup>3-6</sup> frequency with median (range) -0.91% (-2.54%-3.51%,  $p = 0.053$ ) was noted (Figure 1).

### IV Treg Impact for Progression-Free and Overall Survival

Age, response to treatment, bulky lymphadenopathy, *IGHV* mutational status, poor-risk cytogenetics, refractoriness to fludarabine, Treg<sup>0</sup>, Treg<sup>3</sup>, and Treg<sup>6</sup>, as well as Treg<sup>0-3</sup> and Treg<sup>0-6</sup> values were tested as prognostic factors for PFS and OS in Cox regression models. Univariate Cox regression analysis revealed that only a smaller reduction of Treg<sup>0-3</sup> was statistically significantly associated with shorter PFS. Refractoriness to treatment and lower Treg<sup>0</sup> were also associated with shorter PFS, but statistical significance was not reached (Table 1).

Treatment failure, lower Treg<sup>0</sup> frequency, less pronounced reduction of Treg<sup>0-3</sup> and Treg<sup>0-6</sup> were statistically significantly associated with shorter OS in univariate Cox regression analysis (Table 1). Multivariate analysis revealed that clinical treatment failure (HR: 5.472, 95% CI: (1.634-18.331),  $p = 0.006$ ) and less pronounced reduction of Treg<sup>0-3</sup> (HR: 1.342, 95% CI: 1.056-1.704,  $p = 0.016$ ) remained independently statistically associated with shorter OS.

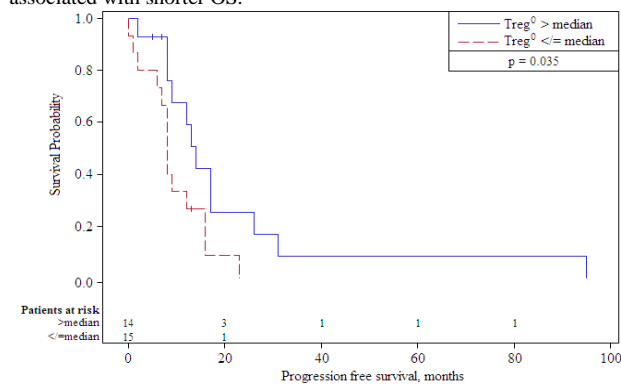


Figure 2: Progression-free survival according to baseline Treg.

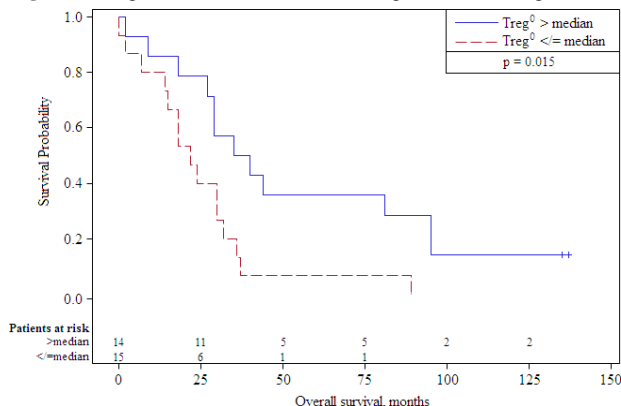


Figure 3: Overall survival according to baseline Treg.

We divided patients into two subgroups according to their median value of Treg<sup>0</sup> (3.70) and Treg<sup>0-3</sup> (2.14). The median (95% CI) PFS in lower and higher/or equal than median Treg<sup>0</sup> was 8.0 (2.0-12.0) and 14.0 (8.0-26.0),  $p = 0.035$ , respectively (Figure 2). Also, the median (95% CI) OS in lower and higher/or equal than median Treg<sup>0</sup> was 22.0 (7.0-30.0) and 37.5 (18.0-95.0),  $p = 0.015$ , respectively (Figure 3). Treg difference median between screening and the three treatment courses did not have impact on PFS (Figure 4), but a deeper reduction of Treg median during the first three courses correlated with better OS (Figure 5).

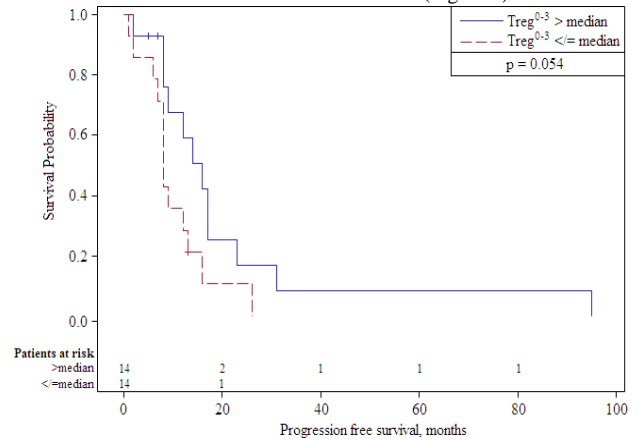


Figure 4: Progression-free survival according to Treg difference median between screening and the three treatment courses.

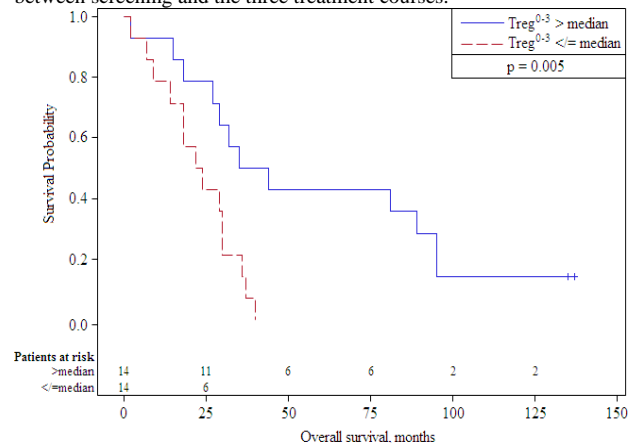


Figure 5: Overall survival according Treg difference median between screening and the three treatment courses.

### Discussion

Increased circulating Treg cells have been identified in untreated CLL patients with respect to healthy subjects in a number of clinical studies [1, 10, 11]. A higher frequency of such cells was shown to be associated with more advanced disease and correlated with higher lymphocyte count and elevated LDH [12, 13]. Treg frequency was not found to correlate with prognostic factors such as ZAP70, CD38 expression, *IGHV* mutational status, or adverse cytogenetic, but little is known about the prognostic value of Treg cells in treated CLL patients [13]. Weiss *et al.* reported data suggesting that circulating Treg cell number may predict the need for treatment in low-intermediate risk CLL patients, concluding that higher percentage of Treg was associated to a shorter time to first treatment [10]. Treatment with some drugs such as fludarabine or lenalidomide led to a reduction in Treg numbers [11, 14].

A normalization in their number was observed after treatment with fludarabine, despite an initial transient increase [11]. The same phenomenon Lee *et al.* reported in 24 out of 60 patients with CLL in whom the detection of Treg cells was performed at study entry, after 3 and after 15 cycles of treatment with lenalidomide [15]. In particular, a significant increase of Treg cells was observed after 3 cycles of therapy, while a normalization was found after the treatment. These data suggest that lenalidomide is able to modulate cell-mediated immunity in patients with CLL. Novel agents as BTK inhibitor ibrutinib were shown to significantly reduce Treg cell subset during the first month of therapy, but the impact on clinical outcome was not evaluated so far [16]. Feuchtenberge *et al.* suggested that Rtx did not change Treg numbers in immune disorders such as rheumatoid arthritis [17]. However, Rtx was shown to normalize low baseline Treg frequency in immune thrombocytopenia, suggesting that defective T regulatory cell compartment can be modulated by a B cell-targeted therapy in some immune disorders [18]. Higher methylprednisolone doses significantly increased T cell apoptosis in autoimmune disorders suggesting better

disease control in an animal model [19]. HDMP and Rtx were shown to be effective in relapsed high-risk CLL patients, especially with adverse cytogenetics [2, 20, 21].

We evaluated T regulatory cells in relapsed/refractory CLL patients treated with HDMP and Rtx combination and their impact on PFS and OS. Screening Treg frequency did not correlate with known adverse prognostic factors such as bulky lymphadenopathy, fludarabine refractoriness, high-risk cytogenetics, and response quality, but lower Treg<sup>0</sup> patients had a trend for shorter PFS and significantly reduced OS (Table 1). There was a significant decrease of Treg frequency after three treatment courses (Treg<sup>0-3</sup>) and at the end of therapy (Treg<sup>0-6</sup>) with a slight progression of Treg<sup>3-6</sup> (Figure 1). This early reduction of Treg was the only factor associated with longer PFS (Table 1). Early reduction of Treg<sup>0-3</sup> was also correlated with prolonged OS. Other factors negatively influencing OS were refractoriness to study treatment, low baseline Treg, and less pronounced reduction of Treg<sup>0-6</sup> (Table 1).

**Table 1:** Univariate Cox regression analysis of prognostic factors predicting PFS and OS.

Characteristics	Progression free survival		Overall survival	
	HR (95%CI)	<i>p</i> value	HR (95%CI)	<i>p</i> value
Male gender	1.355 (0.591-3.106)	0.472	0.813 (0.378-1.750)	0.597
Fludarabine refractoriness	1.984 (0.795-4.947)	0.142	0.897 (0.389-2.067)	0.798
<i>IGVH</i> mutated	0.278 (0.062-1.236)	0.093	0.695 (0.239-2.017)	0.503
Bulky lymphadenopathy*	1.767 (0.754-4.141)	0.190	1.260 (0.580-2.735)	0.559
del17p	1.146 (0.519-2.529)	0.736	1.300 (0.600-2.816)	0.506
del11q	0.955 (0.411-2.219)	0.915	0.597 (0.272-1.313)	0.200
Refractoriness to study treatment	2.188 (0.949-5.042)	0.066	2.536 (1.148-5.604)	0.021
Lower baseline CD4+CD25+CD127-/bl FoxP3+	1.179 (0.997-1.393)	0.054	1.172 (1.009-1.362)	0.038
Lower reduction of CD4+CD25+CD127-/bl FoxP3+ (delta 0-3 months)	1.209 (1.003-1.458)	0.046	1.314 (1.073-1.610)	0.008
Lower reduction of CD4+CD25+CD127-/bl FoxP3+ (delta 0-6 months)	1.071 (0.866-1.290)	0.530	1.205 (1.003-1.447)	0.046

\*Lymphadenopathy > 5 cm.

**Table 2:** Multivariate Cox regression analysis of prognostic factors predicting overall survival.

Characteristics	HR (95%CI)	p
Refractory disease (SD, PD)	5.472 (1.634-18.331)	0.006
CD4+CD25+CD127-/b1 FoxP3+, 0 months.	-	N.S.
delta 0-3 months. CD4+CD25+CD127-/b1 FoxP3+	0.745 (0.587-0.947)	0.016
delta 0-6 months CD4+CD25+CD127-/b1 FoxP3+	-	N.S.

N.S.: Not Significant.

In multivariate analysis, treatment failure and less pronounced reduction of Treg<sup>0-3</sup> remained significant prognostic factors for shorter OS (Table 2). Patients with higher than median Treg<sup>0</sup> had better PFS and OS compared with lower/equal baseline Treg (Figures 2 & 3). A deeper reduction of Treg<sup>0-3</sup> median had a positive impact on OS (Figure 5) but not PFS (Figure 4). Our results suggest that not only baseline T reg frequencies might have an impact on prognosis, but Treg dynamics during therapy could be a more important factor for survival.

In conclusion, our data demonstrated for the first time that high dose methylprednisolone and rituximab combination is effective in reducing T regulatory cells in pretreated high-risk CLL patients and suggest that early decrease and deeper reduction of Treg can be an important factor predicting clinical outcome. Our results should be confirmed in larger studies.

### Conflicts of Interest

None.

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