Pathol. Oncol. Res. (2011) 17:249–255
DOI 10.1007/s12253-010-9306-2

RESEARCH

Pretreatment T Lymphocyte Numbers Are Contributing to the Prognostic Significance of Absolute Lymphocyte Numbers in B-cell Non-Hodgkins Lymphomas

Lajos Gergely · Andrea Váncsa · Zsófia Miltényi · Zsófia Simon · Sándor Baráth · Árpád Illés

Received: 10 April 2010 / Accepted: 1 September 2010 / Published online: 15 September 2010

Abstract Targeted immuno-chemotherapy resulted in greatly improved survival of B cell lymphoma patients. Several prognostic markers are investigated, amongst them the pretreatment absolute lymphocyte numbers. We investigated lymphocyte subpopulations and correlated this data with prognosis of patients. 88 patients (mean age: 56 years, 18–88, median follow up 32 months) with B cell lymphomas were investigated. There were 51 DLBCL, 16 Follicular NHL, 4 MALT, 7 Marginal Zone NHL, 10 Small lymphocytic cases were investigated. Our data showed that overall survival was statistically significant up to the 0.9 G/l absolute lymphocyte numbers as dividers between the subgroups. The CD19+ B cell numbers, or the CD56+/CD3- NK cell numbers did not represent any significant differences between subgroups. However CD3+, CD4+ and CD8+ T cells were differentiating statistically significant subgroups. Pretreatment CD3+ cell number less than 700/ul and CD8+ cell number less than 200/ul were corresponding with significantly inferior overall survival. These could be verified in the bad prognostic IPI group as well. Our data further support the importance of pretreatment lymphocyte numbers and highlight CD3+ and CD8+ lymphocytes to be the key factors in predicting outcome.

Keywords NHL · Lymphocytes · T cells · Prognostic factors

L. Gergely (✉) · A. Váncsa · Z. Miltényi · Z. Simon · Á. Illés
3rd Department of Internal Medicine, Institute of Internal Medicine, DEOEC, University of Debrecen,
Moricz zs. Krt. 22,
4032 Debrecen, Hungary
e-mail: lgergely@iiibel.dote.hu

S. Baráth
Regional Immunology Laboratory, Institute of Internal Medicine, 3rd Dept. of Medicine, DEOEC, University of Debrecen, Debrecen, Hungary

A. Váncsa
Department of Rheumatology, Institute of Internal Medicine, DEOEC, University of Debrecen, Debrecen, Hungary

Introduction

A significant number of B-cell non-Hodgkin lymphoma patients could be cured today with the appearance of monoclonal antibodies, especially rituximab. The precise understanding of the host's immune system and underlying mechanisms is necessary to fully utilize its potential and improve results with the addition of immune therapy. However the basis of the therapy is still the semi- or non selective chemotherapy combined with the immune therapy. Prognostic factors and response indicating factors has been extensively studied for decades now, and numerous well established prognostic models persist, based on tumor type, size and burden. Recently genetic aberrations detected by FISH or PCR could further characterize lymphomas and their possible prognosis. However significantly less knowledge is available regarding the lymphoma's surrounding, the self immune system. The wide administration of rituximab and other monoclonal antibodies resulted in increased interest in hosts immune status, immune reaction against the lymphoma.

Lymphocytes as key elements of the immune system play a substantial role in this whole complex system. Malignant lymphomas involving the B cell types are of great importance, because the possibility to administer the very effective rituximab antibody with the conventional chemotherapy. Further characterizing the cell of origin in lymphomas may provide additional insight into pathogenesis and prognosis [1]. The first publication was in 1972 by

Marini in Hodgkins disease [2], but not until 2003 this was investigated again. Decaudin and collegues reported that the pretreatment absolute lymphocyte numbers correlated with outcome of monoclonal anti-CD20 treatment [3]. Examining a large cohort of follicular lymphoma patients, the absolute lymphocyte count proved to be an independent prognostic factor, besides the FLIPI [4]. Since then several papers were published in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) patients proving the pretreatment absolute lymphocyte numbers to be prognostic factors. A cutoff value of 1.0 G/l is generally proved to be the margin, however some authors report 0.9 G/L as cutoff. This is a very useful, and easily reproducible test yielding important results. However the subset of lymphocytes involved in this mechanism is not fully characterized yet. There is data emphasizing CD3+ T lymphocytes to be the key elements of this value [5]. Further characterizing the T cells, lymph node infiltrating CD8+ cells seems to be correlating with prognosis [6].

Additional data is provided in this paper regarding lymphocyte subsets in the prognosis of B cell non-Hodgkin lymphomas.

Design and Methods

From the 1st of September 2002 to the 1st of April 2008, 112 patients with B-cell non-Hodgkin lymphoma treated in our institution were selected randomly. The study was approved by the IRB as no additional procedures were required than routine bloodwork, so no informed consent is required. Sufficient follow up data was available from 88 patients, and data was reported on these cases. There were 57 female and 31 male patients, mean age at diagnosis was 56 years [range 18–88 years]. The median follow up time was 32 months [range: 10–76 months]. Histological subtypes were as follows: 51 Diffuse Large B-Cell Lymphoma (DLBCL), 15 follicular NHL, 10 small lymphocytic lymphoma, 7 marginal zone lymphoma (nodal) and 4 Mucosa Associated Lymphoid Tissue (MALT) lymphoma. All cases were examined by 3 color flow cytometry and had no detectable circulating lymphoma cells in the peripheral blood. The routine staging consisted of bone marrow biopsy and aspirate for flow, CT scans of neck-thorax, abdomen and pelvic regions with additional ultrasound of peripheral lymph nodes. PET/CT was only used to confirm residual masses in all cases, and was used in every case for staging as well since March 2007. Complete renission (CR) was stated only when no active tumor was found, as described by the Cheson criteria. Distribution according to the Ann Arbor stage is as follows: stage I – 8; stage II – 16; stage III – 23; stage IV – 41. Altogether there were 27 patients with B symptoms. Standard treatment regimens were 8x R-CHOP 21 for DLBCL and follicular NHL, 6-8x CHOP 21 for marginal zone and MALT, 6-8x CVP or CHOP for small lymphocytic lymphoma. In case of bulky disease at presentation, involved field irradiation (30–36 Gy) was given after chemotherapy. Patients not receiving rituximab for first line did receive rituximab for second line regimens if needed. Sixty two patients responded well to 1 therapeutic regimen, not needing additional therapy, and 26 patients received more than 1 regimen, mostly for relapse/progressive disease (Table 1).

Routine blood analysis was done as part of the treatment protocol on automated blood counter. All blood count

Table 1 Characteristics of patients involved in the study

Mean age of patients:	56 years [16–88]
Mean follow up time:	32 months [10–76]
Histologic subtypes:	No. of patients
DLBCL	51
Follicular NHL	16
Small lymphocytic lymphoma	10
Marginal zone lymphoma (nodal)	7
MALT lymphoma	4
Ann Arbor stage:	
I	8
II	16
III	23
IV	41
B symtpoms	27
First line therapy:	
R-CHOP	66
CHOP	16
CVP	6
Response to first line:	
CR	73
PR	10
PD/death	5
Relapse after CR/PR:	26
IPI score	
0	12
1	27
2	31
3	10
4	8

Patient characteristics

DLBCL diffuse large B-cell lymphoma, *NHL* non-Hodgkin's lymphoma, *MALT* mucosa associated lymphoid tissue, *R-CHOP* rituximab, cyclophosphamide, adriablastina, vincristine, prednisolone chemotherapy, *CVP* cyclophosphamide, vincristine, prednisolone chemotherapy, *CR* complete remission, *PR* partial remission, *PD* progressive disease, *IPI* International Prognostic Index

samples were drawn at the same time with heparinized samples.

Flow cytometry analysis was done with the following reagents: Dako MultiMix™ CD4/FITC, CD8/RPE, CD3/RPE-Cy5 (Dako Denmark A/S), BD Simultest CD3/FITC, CD16+56/PE (Becton-Dickinson, NJ, USA), and CD19/PC5 (Immunotech, Marseille, France). Heparin anticoagulated whole blood samples were freshly drawn from patients and stained with monoclonal antibodies according to the recommended protocol. Samples were washed, and red cells were lysed and samples were fixed with 1% paraformaldehyde solution. Measurement was done on a Coulter XL4 flow cytometer (Coulter, Hialeah, FL, USA) using the supplied EPICS software and from June 2006 on a Beckman-Coulter CYTOMICS FC500 flow cytometer (Beckman-Coulter, Fullerton, CA, USA) using the supplied CXP software. Gates and analytic regions were set by isotype control antibodies simultaneously stained with samples. Absolute numbers were calculated using routine whole blood count result multiplied by the flow cytometry percentages. Data was collected in Microsoft Excel and statistical analysis was done with Statsoft Statistica v8.0 and SPSS 15 softwares. Kaplan-Meier survival curves are presented and differences between the survival curves were tested with the two tailed log-rank test [7]. Absolute cell numbers were assessed as continuous variables and dichotomized based on the findings of the optimal cutoff point based on the log-rank statistics [8]. Prognostic significance of variables on overall survival (OS) was measured using the Cox proportional hazard model [9]. All p values reported were two-sided, and statistical significance was stated when $p<0.05$.

Results

The characteristics and treatments of 88 patients are reported in Table 1. The majority of patients responded to first line therapy, with high rate of complete remissions (82.9%), however 26 patients (31%) relapsed, requiring additional treatments. The majority of patients (64 patients – 72%) had advanced stage at diagnosis. There were 18 (20.4%) cases with high risk IPI score, and 39 (44.3%) with low risk IPI score.

The standard prognostic factors were also investigated. The overall survival was significantly better in the low risk IPI (0-1) patients, compared to the others ($p=0.001$). Median survival not reached in either group. The low risk IPI group had 97% probability of survival at 4 years compared to 71% for the remaining patients (data not shown). Patients with bulky disease at diagnosis had a significantly inferior overall survival at 4 years with 52% compared to the others with 90% ($p=0.001$) (data not shown). Patients with elevated LDH had significantly worse overall survival at 4 years with 61% compared to the other group where overall survival (OS) was 88% (HR: 0.152, 95%CI: 0.047–0.491, $p=0.002$) (data not shown). These represent that the standard prognostic factors were well differentiating our patients as well.

It has been reported that B2-microglobulin is a good prognostic marker of inflammation, and has a strong predictive role in CLL patients [10]. It also proved to be a good prognostic marker in our patients, as the OS at 4 years was 95% when the B2-microglobulin was normal, compared to a 4 year OS of 76% in the cases with elevated levels (HR: 0.275, 95%CI: 0.061–1.244, $p=0,03$) (data not shown).

The pretreatment absolute lymphocyte numbers were investigated, and a cutoff value of 0.9 G/L was found to be the threshold for significantly (HR: 0.223, 95% CI: 0.083–0,597, $p=0.0039$) differentiating overall survival between groups. Patients with greater than 0.9 G/L lymphocytes had a 4 year overall survival (OS) of 90% compared to 52% in the other group. We could not confirm the cutoff value of 1 G/L for OS difference as $p=0.129$ was calculated. This was also confirmed for Event Free Survival, where the 1 G/L absolute lymphocyte number was a cutoff with significance ($p=0.0498$) (data not shown).

Further analyzing the lymphocyte subgroups of T-B-NK cells we could confirm that T cells are the most sensitive prognostic markers. The average CD19+ B cell pretreatment value was 0.26 G/L (SD:0.25). We could detect a statistically different ($p=0.0475$) overall survival function with a cutoff of 0.15 G/L, but this was not valid neither for 0.1 G/L nor for 0.2 G/L, suggesting not a valid marker. The average CD16+56+ and CD3- NK cell number before treatment was 0.22 G/L (SD:0.12). Patients with less then 0.05 G/L pretreatment NK cells had worse prognosis but this was not statistically significant. Also we could see a non significant observation, that patients with higher (>0.3 G/l) pretreatment NK cell numbers have a slightly inferior overall survival, but only after 4 years. Mean absoulte CD3+ number before treatment was 0.99 G/L (SD: 0.44). Significant difference in predicted overall survival was from 0.7 G/L absolute CD3+ cell numbers (HR: 0.317, 95%CI: 0.121–0.827, $p=0.0246$) (Fig. 1). By lowering the threshold to 0.5G/L the worse prognostic group can be better identified ($p=0.0075$), as 4 year predicted OS was 65% with 0.7 G/L CD3+ cells cutoff and was 55% with 0.5 G/L CD3+ cells cutoff (data not shown).

Dividing the CD3+ cells into CD4+ and CD8+ subgroups, further analysis was done. The mean pretreatment CD4+ cell number was 0.62 G/L (SD: 0.27) and the CD8+ cell number was 0.31 G/L (SD: 0.17). Examining the pretreatment absolute CD4+ cell numbers we could not identify a threshold that significantly differentiates between

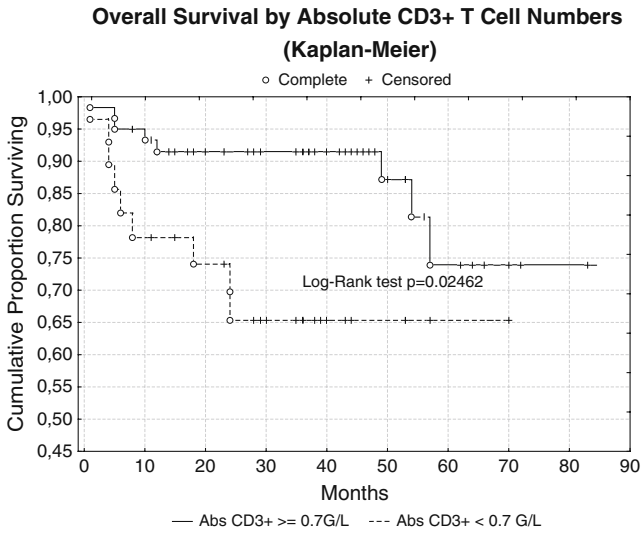

Fig. 1 Overall survival by absolute CD3+ T cell numbers (Kaplan-Meier)

good and bad prognostic groups. Patients having less than 0.5 G/L pretreatment CD4 cells tend to have inferior outcome (4 year OS 70%) compared to the high CD4 group (4 year OS 88%), but this could not be statistically proved (p=0.0984) (data not shown). By lowering this threshold to 0.3 G/L still no significant difference could be obtained. On the opposite the CD8+ cell numbers could predict overall survival. Lower than 0.2 G/L absolute CD8+ cells before treatment clearly differentiated a worse prognostic group with 4 year OS of 69% in contrary to the 92% of the remaining patients (HR: 0.303, 95%CI: 0.111–0.822, p=0.0147) (Fig. 2).

To investigate if these pretreatment lymphocyte subset values have any additional value in differentiating within IPI prognostic groups we separated our patients into IPI 0-1 'good prognostic' and IPI > 2 'bad prognostic' groups and investigated the additional information given by these values.

In the good prognostic group patients with greater than 0.8 G/L pretreatment CD3+ cells had a better overall survival 5 years 100%, but this was not statistically different to the remaining patients, where the 4 year OS was 96%, but 5 year OS was 86% (p=0.55) (data not shown). The absolute pretreatment CD8+ cells below 0.15 G/L could however differentiate an inferior subgroup, but this was still not statistically significant (p=0.149). Only 2 patients had a pretreatment CD4+ cell number lower than 0.3 G/L, so a higher threshold of 0.5 G/L was investigated where there were 10 patients in the low CD4+ cell number group. No significant difference was found between these groups.

In contrary the bad prognostic group could be further divided by the absolute CD3 cell number. With a cutoff value of 0.8 G/L the difference was not significant (p= 0.213), but lowering the value to 0.5 G/L clearly defined two distinct groups, with statistically different overall survival (Fig. 3). Patients with less than 0.5 G/L CD3+ cells at diagnosis had a 45% 2 year OS comperd to 83% in the remaining patients (p=0,0398). Pretreatment CD8+ cell numbers lower than 0.3 G/L were clearly differentiating an inferior outcome subgroup with 4 year OS of 60%, compared to 84% in the remaining patients (Fig. 4), but this is not statistically significant (p=0.0857). Lower than 0.3 G/L CD4+ cells indicated an inferior outcome with 4 year OS of 52% compared to 80%, but this was not statistically significant (p=0.1807).

Analyzing these results in the individual histologic groups the following could be confirmed. There were 51

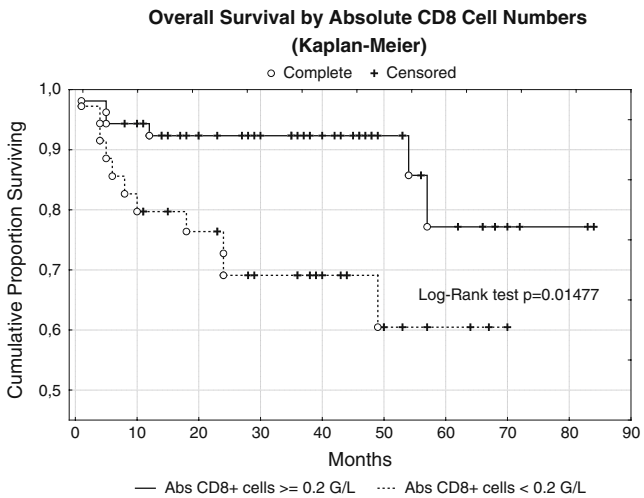

Fig. 2 Overall survival by absolute CD8 cell numbers (Kaplan-Meier)

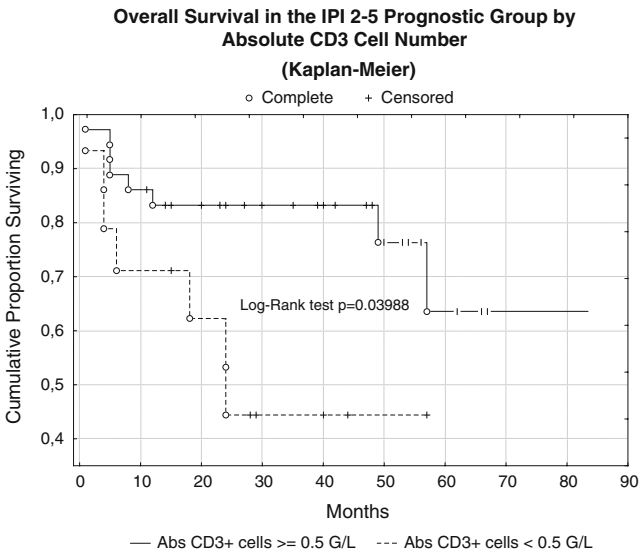

Fig. 3 Overall survival in the IPI 2-5 prognostic group by absolute CD3 cell number (Kaplan-Meier)

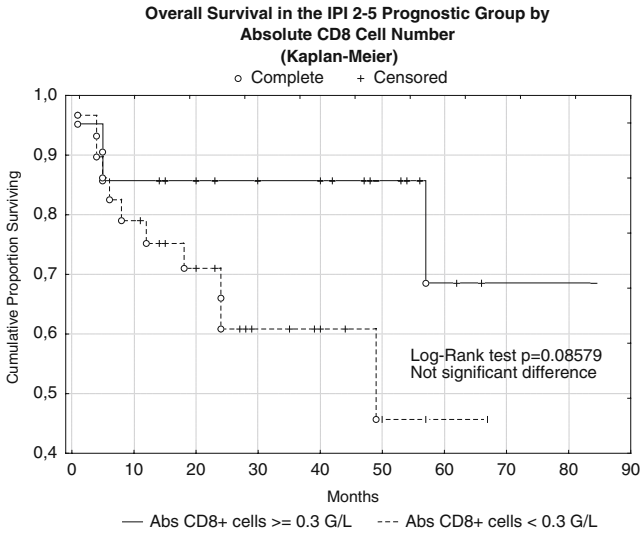

Fig. 4 Overall survival in theIPI 2-5 prognostic group by absolute CD8 cell number (Kaplan-Meier)

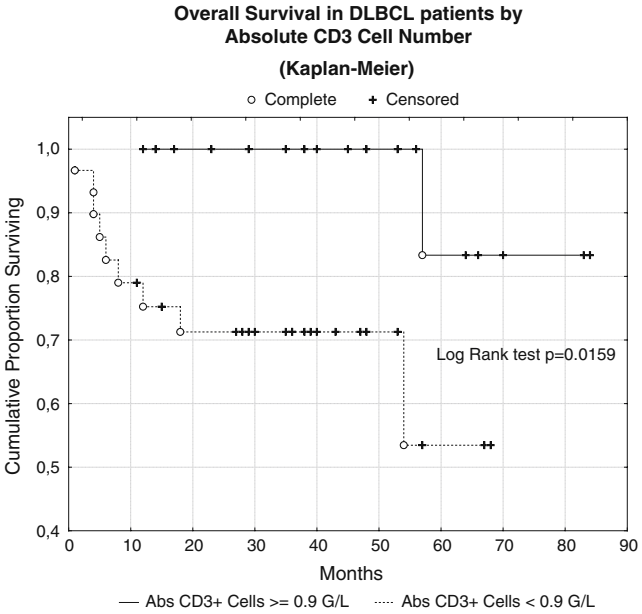

Fig. 5 Overall survival in DLBCL patients by absolute CD3 cell number (Kaplan-Meier)

DLBCL cases (mean age: 54.98 years, range: 18–88 years, 27 females, 24 males) included in the study. Absolute CD3 numbers were not significantly differentiating the 2 subgroups at a cutoff value of 0.7 G/l as indicated for all patients (p=0.082), however there was a clear benefit for patients having higher CD3+ cells numbers for the first 4 years, but this disappeared later (data not shown). However by increasing the cutoff to 0.9 G/L a clearly significant difference was found (p=0.0159) favouring patintes with higher pretreatment CD3+ cell numbers. As indicated on the graph patients had a 100% chance for 4 year survival with high CD3+ cells (Fig. 5). Analyzing the CD8+ cell numbers we could confirm the cutoff value of 0.3 G/L significantly differentiating prognostic subgroups (p=0.0456) (Fig. 6). Analyzing the pretreatment B cell numbers a difference was found from the whole chort, as a cutoff value of 0.2 G/L was clearly differentiating better prognosis with higher B cell numbers (p=0.0435).

There were 16 Follicular lymphoma cases (mean age: 57.57 years, range: 37–83 years, 12 females and 4 males). Examining the pretreatment CD3+ cell numbers, we could not confirm the validity of the 0.9 G/L cutoff (p=0.685). By decreasing the cutoff to 0.7 G/L we could still not confirm significance (p=0.779) Analyzing the CD8+ cells we could not confirm the 0.3 G/L cutoff to be significantly differentiating prognostic groups (p=0.223) Analyzing the CD19 cells, no cutoff was found, and with the shown 0.2 G/L cutoff value there was no difference (p=0.918). It should be noted however, that due to the small sample size, the statistics are not significant, suggesting that this has to be analyzed in larger population.

The remaining histologic subgroups had even less patients, so data was not analyzed separately.

Using Cox regression analysis however we could not confirm that CD3+, CD4+ or CD8+ cells differentiate statistically different and independent subgroups. (data not shown) Only the absolute lymphocyte numbers were independent of IPI. Examining LDH levels and β2-microglobulin levels, we also could not confirm that CD3+, CD4+ or CD8+

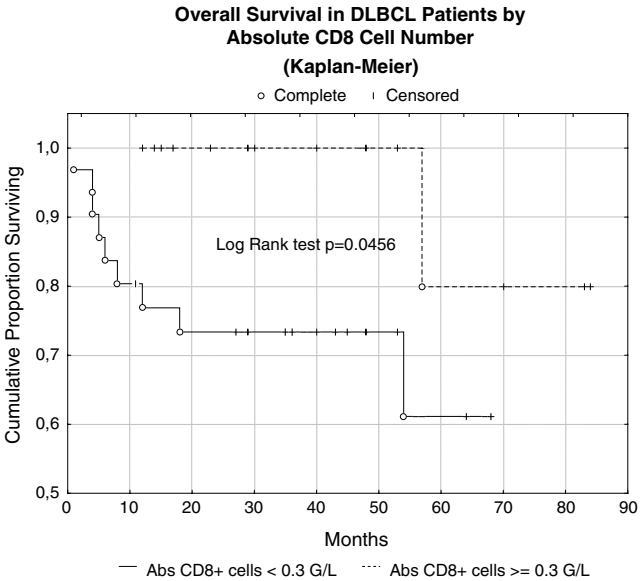

Fig. 6 Overall survival in DLBCL patients by absolute CD8 cell number (Kaplan-Meier)

cells different statistically independent subgroups (data not shown). This may be due to the relative smaller number of patients in each subgroup.

Discussion

Prognostic markers are very important for treating physicians to best predict response and choose therapy. They have to be easily reproducible tests available in all hospitals. IPI and its variations and newer factors as β2 microglobulin are very important. During the last 3 years several reports highlighted the importance of pretreatment absolute lymphocyte numbers [11–14]. It has been shown that patients having less than 0.9 G/L pretreatment absolute lymphocytes bear an inferior survival. This was proven to be an independent prognostic factor in follicular lymphoma and DLBCL [4, 13]. It has been validated as having prognostic value in Hodgkin's lymphoma [15] and acute myeloid leukemias as well [16]. It is very important to further categorize lymphocyte populations, as it is a dynamically changing population, consisting of several major cell types. By further subdividing lymphocytes into subsets with a flow cytometer and routine technique an even more precise predictive factor could be established. To date only Czuczman and collegues provided data regarding the important lymphocyte population to be the CD3+ T cells in follicular lymphoma patients [5]. They also emphasized that with the addition of rituximab to standard chemotherapy the conventional prognostic factors were not as strong predictors as newer factors like CD3+ T cells, LDH level, B2 microglobulin.

The data presented in the paper clearly gears the focus on T cells, as no association is found between overall survival and B or NK cells. This finding corresponds with the available data [5]. When subdividing T cells into CD4 and CD8 groups, only the CD8+ cells corresponded significantly with overall survival. This is a novel data, only proven in lymph nodes but not in peripheral blood before [6]. Patients with low CD8+ cell numbers (less than 0.2 G/L) had a significantly inferior overall survival, and this could even be proven in the low risk IPI group. This is a very interesting data, clearly shifting our focus towards CD8+ T cells. Constituting the majority of effector immune cells, their absence may result in reduced cell mediated killing and apoptosis induction through cell-cell binding. This data is partially supported by the promising results with in vitro expanded and primed autologous LAK cell therapies in malignancies [17]. Another possible explanation can be the altered cytokine profile in the patients, resulting in unfavourable Th2 shift and suppressing Th1 like effector mechanisms and CD8+ cell numbers [17].

A reasonably heterogenous population was investigated in the paper, similar to what is found in everyday practice. The common factor was that all were CD19, CD20 B cell malignancies and had no detectable circulating tumor cells. By further subdividing patients, slightly different cutoff values could have been achieved, but this was not our goal for this report. Howevere we provided data on DLBCL and Follicular Lymphoma patients, highlighting that slight variances can be in the cutoff values by each subgroup. The larger patient population of DLBCL cases correlated well with the whole population for obvious reasons.

By proving the validity of the CD3 and CD8 cell numbers in a mixture of B cell lymphoma patients we could adopt it in everyday practice, for the follow up and possibly later in the treatment of patients. Our study group is not too large, so statistical methods have limitations in interpreting data, but by confirming already established factors, we could validate the study group and tests used.

We conclude that patients regardless of IPI or other prognostic factors having less than 0.2 G/L CD8+ T cells at diagnosis or having less than 0.5 G/L CD3+ T cells at diagnosis are having an inferior overall survival, necessitating dose dense therapy and possibly no cycle reductions. These patients should be further investigated for any possible methods to restore their skewed immune balance.

Authorship and Disclosions No conflict of interest is reported by the authors.

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