CD4:CD8 T-Cell Ratio Differs Significantly in Diffuse Large B-Cell Lymphomas from Other Lymphoma Subtypes Independently from Lymph Node Localization

Cécile Daussy1,2,3, Diane Damotte1,3,4,5, Thierry J. Molina3,4, Mikael Rousselet6, Thierry Fest6,7, Audrey Varin1,2,1, Jean-Yves Perrot8, Lamia Ouattara1,3,4, Hélène Merle-Béal9, Pierre Julia10,11, Wolf Herman Fridman1,2,3, Catherine Sautès-Fridman1,2,3, and Sylvain Fisson1,2,3,11,12,13,*

Abstract—Although the identification of newly described T-cell subpopulations in human B-cell lymphoma is under investigation, contradictory data still persist regarding the relative proportions of CD4+ and CD8+ T-cells in tumoral lymph nodes (LN). CD4:CD8 T-cell ratio in LN from Hodgkin (HL) and non-Hodgkin B-cell lymphoma subtypes at diagnosis was analyzed by flow cytometry based on three independent cohorts (n=112). A significantly lower median CD4:CD8 ratio (0.82±1.06) was observed only in diffuse large B-cell lymphomas (DLBCL). Ratios were unchanged by the localizations suggesting that the lymphoma subtype influences the relative proportions of CD4+ and CD8+ T-cells in the tumoral LN.

Index Terms—CD4:CD8 T-cell ratio, B-cell lymphoma, Hodgkin lymphoma, lymph node, flow cytometry

Abbreviations—DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HL, Hodgkin lymphoma; LL, lymphocytic lymphoma; LN, lymph node; NHL, non-Hodgkin B-cell lymphoma; Tc, cytotoxic T-cells; Th, helper T-cells; TIL, tumor infiltrating lymphocytes; Treg, regulatory T-cells.

I. INTRODUCTION

INTEREST regarding the importance of the local adaptive immune microenvironment in cancer is growing [1-3]. Although CD4 and CD8 respective proportions and function were reported as predictors of patient outcome in cancer, studies were often unclear or contradictory in lymphomas [4-7]. Some data suggest that increased numbers of cytotoxic CD8+ T-cells could be an unfavourable marker [8]. Some of these controversial results could be due to the heterogeneity of the lymphoma subtypes and lymph node (LN) localization, methods of study, reagents used and time of analysis (ie. before or after treatment) [6-7]. Our aim was to analyze extensively by flow cytometry the CD4:CD8 T-cell ratio in LN from Hodgkin lymphomas and different subtypes of non-Hodgkin B-cell lymphomas at diagnosis and to compare them to normal and reactive LN. 113 LN from three independent cohorts of 112 untreated patients were analyzed both by flow cytometry and histopathology, and were classified in 6 entities: normal (N); reactive (R); Hodgkin Lymphoma (HL); lymphocytic lymphoma (LL); follicular lymphoma (FL); diffuse large B-cell lymphoma (DLBCL). The cohorts included 24 primary mediastinal HL and DLBCL. Our results clearly showed a lower CD4:CD8 ratio in DLBCL, including the mediastinal localization, compared to other lymphomas subtypes.

II. MATERIALS AND METHODS

A. Patients and controls

Control and tumoral LN were selected at diagnosis from three independent cohorts (Table 1). This study was performed in accordance with the Declaration of Helsinki. All data were treated confidentially. The study was performed with the approval of hospital review boards. All specimens were analyzed in the pathology department from Paris-HEGP, Paris-Hôtel Dieu, and Rennes-Pontchaillou hospitals and were reviewed blindly by expert hematopathologists (DD, TJM). Normal LN corresponded to intra-thoracic non-inflammatory and non-tumoral LN harvested during cardiac surgery (n=11,
cohort #1). Reactive LN were non tumoral but inflammatory or adjacent to a lymphomatous LN for one DLBCL patient (n=7, cohort#1). Tumoral LN were HL, LL, FL or DLBCL (n=27, cohort#1; n=22, cohort#2; n=46, cohort#3).

### TABLE I
**SUMMARY OF PATIENT CHARACTERISTICS AT DIAGNOSIS FOR COHORTS #1 (PARIS-HEGP HOSPITAL), #2 (PARIS-HÔTEL DIEU HOSPITAL), AND #3 (RENNES-PONTCHAULI HOSPITAL).**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Lymph node</th>
<th>Number (% of total)</th>
<th>Age Min-max (median)</th>
<th>Sex M/F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>11 (26.7%)</td>
<td>18-81 (72)</td>
<td>5/2</td>
</tr>
<tr>
<td>#1</td>
<td>Reactive</td>
<td>7 (6.2%)</td>
<td>25-70 (32)</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>HL, non mediastinal localizations</td>
<td>7 (6.2%)</td>
<td>21-80 (35)</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>4 (3.5%)</td>
<td>64-86 (75)</td>
<td>4/0</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>9 (8%)</td>
<td>27-85 (56)</td>
<td>5/4</td>
</tr>
<tr>
<td></td>
<td>DLBCL, mediastinal</td>
<td>2 (1.8%)</td>
<td>25-55 (40)</td>
<td>2/0</td>
</tr>
<tr>
<td></td>
<td>Other localizations</td>
<td>5 (4.4%)</td>
<td>53-86 (73)</td>
<td>3/2</td>
</tr>
<tr>
<td>#2</td>
<td>DLBCL, mediastinal</td>
<td>10 (9.9%)</td>
<td>18-86 (33)</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Other localizations</td>
<td>4 (3.5%)</td>
<td>40-79 (65.5)</td>
<td>4/0</td>
</tr>
<tr>
<td></td>
<td>HL, mediastinal</td>
<td>7 (6.2%)</td>
<td>25-80 (32)</td>
<td>4/3</td>
</tr>
<tr>
<td></td>
<td>Other localizations</td>
<td>4 (3.5%)</td>
<td>64-86 (75)</td>
<td>4/0</td>
</tr>
<tr>
<td>#3</td>
<td>DLBCL, mediastinal</td>
<td>10 (9.9%)</td>
<td>18-86 (33)</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Other localizations</td>
<td>4 (3.5%)</td>
<td>20-25 (23.5)</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>HL, mediastinal</td>
<td>1 (0.9%)</td>
<td>25-80 (32)</td>
<td>6/3</td>
</tr>
<tr>
<td></td>
<td>Other localizations</td>
<td>15 (13.3%)</td>
<td>15-80 (32)</td>
<td>6/9</td>
</tr>
</tbody>
</table>

HL, hodgkin lymphoma; LL, lymphocytic lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; M, male; F, female.

### B. Cell isolation

Fresh unfixed LN were minced with a sterile blade in RPMI medium and were either digested with 2mg/mL Collagenase (Worthington, Roche, Meylan, France) and 0.1mg/mL Dnase I (Roche, Meylan, France) at 37°C for 50min, filtered and rinsed in PBS with 2mM EDTA and 2% FCS (cohort#1), or were subjected to a mechanical dissociation in petri dishes using 25-gauge needles (cohort#2), or with the Medimachine (Becton Dickinson, San Jose, CA) (cohort#3).

### C. Flow cytometry

Cells were preincubated with PBS + 2% human AB serum to block non-specific binding to Fc receptors, then 5.10⁵ cells per well were stained with ECD anti-CD3 (UCHT1), PE-cyanin5 anti-CD4 (13B8.2), PE-cyanin7 anti-CD8 (SFC121Th2D3) from Beckman Coulter, or with PE-cyanin5 conjugated anti-CD3 (UCHT1, Dako, Trappes, France) and/or with the following BD-Biosciences (Le Pont-de-Clair, France) mAb: Alexa fluor 700 anti-CD3 (UCHT1), PE anti-CD4 (SK3), Pacific blue anti-CD4 (SK3), FITC anti-CD8 (SK1), APC-cyanin7 anti-CD8 (SK1) or the corresponding isotypic mAb controls. Data were acquired using a nine-colours LSRII (BD-Biosciences) or a four-colors FACS caliber (BD-Biosciences) or a five-colors FC500 (Beckman Coulter), and analyzed with the Diva (BD-Biosciences), Cellquest (BD-Biosciences), or CXP softwares (Beckman Coulter) for cohorts #1, #2 and #3, respectively.

### D. Immunohistochemistry

Immunohistochemistry staining was performed on paraffin embedded sections with the following mAb specific to CD3, (clone SP7, Microm Neomarker, Francheville, France), CD8 (114B, Dako), TiA1 (TiA1, Beckman Coulter, Villepinte, France), Granzyme B (GranzB, Tebu, Le Perray-en-Yvelines, France) for the T-cell subtypes. Briefly, immunostaining on paraffin section was performed after antigen retrieval, avidin/biotin and Fc receptors blocking treatment. Staining was revealed with streptavidin-peroxidase and DAB.

### E. Statistical analysis

Mann-Whitney tests were performed using StatView (SAS Institute, Cary, NC). P values less than 0.05 were considered statistically significant.

### III. RESULTS AND DISCUSSION

LN from 7 HL and 21 B-NHL subtypes were analyzed by flow cytometry for the presence and the relative distribution of CD4⁺ and CD8⁺ T-cell subsets. These lymphomas were compared to 11 normal and 7 reactive LN (Table 1, cohort #1). Non tumoral LN as well as HL, LL and FL samples showed a median CD4:CD8 ratio > 2 (Fig. 1). Surprisingly, an inverted median CD4:CD8 T-cell ratio was specifically observed in DLBCL (0.76±0.33; DLBCL vs normal LN: P<0.001).

![Fig. 1. T-cell characterization from normal, reactive and tumoral lymph nodes. Flow cytometric analysis CD4:CD8 T-cell ratio from normal (N), reactive (R), classical Hodgkin lymphoma (HL), lymphocytic lymphoma (LL), follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) lymph nodes for cohort #1 (black or dark-blue symbols), cohorts #2 (open symbols) and cohort #3 (light-blue symbols). The black arrows indicate non tumoral reactive and tumoral lymph nodes from the same DLBCL patient. CD4:CD8 T-cell ratios are represented on a logarithmic ordinate in order to respect the proportionality of distribution between values over or below 1. The horizontal red bar corresponds to the median value. Statistical analysis was performed using the Mann-Whitney test. Significativity values are indicated as follow: *: P < 0.05 ; **: P < 0.01 ; ***: P < 0.001.](image-url)
shown in Fig. 1 (open and light-blue diamonds, cohorts #2 and #3 respectively) confirmed the low median CD4:CD8 ratio (0.79±0.57 and 1.22±1.27, respectively) without statistical difference between the three cohorts (P=0.37). Moreover, in two DLBCL cases, tumoral LN from cohorts #1 and #2 were treated simultaneously but independently with different techniques and the respective CD4:CD8 T-cell ratios were quite similar (data not shown). Interestingly, a tumoral LN and an adjacent non tumoral LN (confirmed by pathological examination) from one patient were analyzed simultaneously and different CD4:CD8 ratios were found (Fig. 1, arrows). In this case, the CD4:CD8 ratio from the LN without presence of tumor cells was in the range of the set of reactive LN whereas the tumoral LN displayed the lowest CD4:CD8 ratio (0.11). Therefore, the low CD4:CD8 T-cell ratio for DLBCL samples seems to be restricted to the tumoral LN and may be induced by the tumour cells. Moreover, the CD4:CD8 ratio is not linked to the T-cell number variation (data not shown).

In an extensive study on T cell lymphoma, Gorczyca et coll. noticed a low CD4:CD8 ratio in 10 DLBCL cases [5]. Another study showed that DLBCL patients displaying the lowest CD4:CD8 ratios showed a worse overall survival, independently from the clinical stage at diagnosis [9]. However, no extensive comparison between normal and tumoral lymphoid tissues, nor between LN from HL and non-Hodgkin lymphoma subtypes was performed in these studies. In the present work we analyzed 67 LN from three independent cohorts classified in 6 entities and we demonstrate that DLBCL LN exhibit an inverted CD4:CD8 ratio before any treatment.

To investigate the influence of the mediastinal (ie. thymic) localisation on the CD4:CD8 ratio, we compared DLBCL and HL which are the most common lymphomas of the mediastinum. Identification of these tumors (DLBCL; HL, respectively) was assessed by immunohistochemistry for the expression of the following markers: CD20⁺ (12/12; 0/7), CD79a⁺ (12/12; 0/7), CD23⁺ (10/12; 1/7), CD30⁺ (10/12; 7/7), CD15⁺ (0/12; 5/7), and LMP-1⁺ (0/12; 1/7). The median CD4:CD8 ratios are below 1 in primary mediastinal (0.69±1.15) and non mediastinal (0.91±1.03) tumoral LN from DLBCL patients, and are not significantly different (Fig. 2; P=0.10). Contrasting with DLBCL, the median CD4:CD8 ratio for HL was over 3, independently from the mediastinal and non mediastinal localisations (3.93±4.29 and 5.61±10.98, respectively; P=0.73). Altogether these results suggest that the CD4:CD8 ratio seems to depend more on the lymphoma subtype than on tumoral LN localization. The CD4:CD8 ratio evaluation in LN could be an interesting additional tool for the differential diagnosis between some primary mediastinal DLBCL and mediastinal HL.

The inverted median ratio in DLBCL could be explained by an increased number of CD8⁺ T-cells, a decreased number of CD4⁺ T-cells, or both. Using immunohistochemistry, we found a high proportion of CD8⁺ cells compared to the CD3 staining of T-cells in DLBCL (Fig. 3). In contiguous slides, these cells expressed cytotoxic proteins like TiA1 and Granzyme B. Moreover, the DLBCL T-cell proportion did not differ from HL and FL, nor reactive and normal LN (data not shown).
IV. CONCLUSION

Altogether, these data suggest an increased number of cytotoxic T-lymphocytes, associated to a decreased number of CD4+ T-cells. Since this inverted median ratio is characteristic of DLBCL, and differs significantly from low grade lymphoma subtypes, the T-cell subset modulations may be involved in the lymphomagenesis processes of DLBCL.

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REFERENCES


