Peripheral blood natural killer cell count is associated with clinical outcome in patients with aaIPI 2–3 diffuse large B-cell lymphoma


On behalf of GELA (Groupe d’étude des lymphomes de l’adulte)

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Background: Lymphocytopenia is a prognostic factor in Hodgkin’s disease. In diffuse large B-cell lymphoma (DLBCL), data are much less established, in spite of numerous reports on immune system–lymphoma interactions. This study addresses the prognostic value of blood lymphocyte subsets at diagnosis in DLBCL.

Patients and methods: Absolute values of blood lymphocyte subsets and monocytes were prospectively determined by flow cytometry in 140 patients with 2 or 3 adverse age-adjusted International Prognostic Index (aaIPI) factors included in a Groupe d’Etude des Lymphomes de l’Adulte protocol (LNH-08B3). Absolute cell counts at diagnosis and aaIPI were evaluated with regard to clinical outcome.

Results: Low median cell counts of 337, 211, and 104/µl were evidenced for the CD4+ , CD8+ T, and natural killer (NK) cells, respectively. In univariate analysis, only NK cell count [odds ratio (OR) = 1.81 (1.27, 2.57), P = 0.001] and aaIPI [OR = 2.29 (0.95, 5.45), P = 0.06] were associated with induction treatment response. Low NK cell count [Hazard ratio (HR) = 1.27 (1.06, 1.52), P = 0.01] and aaIPI [HR = 1.95 (1.20, 3.16), P = 0.01] were also associated with a shorter event free survival (EFS). In multivariate analysis, NK cell count was associated with response [OR = 1.77 (1.24, 2.54), P = 0.002] and EFS [HR = 1.25 (1.04, 1.50), P = 0.02] independently of aaIPI.

Conclusions: This study shows an association between circulating NK cell number and clinical outcome in DLBCL, possibly important in the context of the broadening use of rituximab, a likely NK-dependant therapy.

Key words: Diffuse large B-cell lymphoma, lymphopenia, NK cells, prognosis

introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of lymphoma, accounting for ~40% of lymphoid malignancies [1]. In this heterogeneous condition, the International Prognostic Index (IPI) based on clinical data (age, Ann Arbor tumor stage, serum lactate dehydrogenase (LDH), performance status (PS), and number of extranodal sites) was designed to identify patients at high risk of death [2]. However, a marked degree of heterogeneity in disease course still remains within each IPI group of patients [3]. This has generated a considerable amount of research aimed at discovering other prognostic markers.

A first field of research was aimed at a better understanding of the biology of the tumor itself, including study of cytogenetic abnormalities and their consequences on oncogenes, translating into cell cycle or apoptosis deficiencies and/or involved in B-cell differentiation abnormalities (reviewed in [4]). A second field of investigation was focused on interactions between the host immune system and the tumor. An association between major histocompatibility complex class II expression by the tumor and prognosis was demonstrated [5], as well as between
the lymph node signature of reactive nonmalignant cells and favorable outcome [6]. However, research on tumor-infiltrating lymphocytes led to various, sometimes controversial results depending on studied subsets [7, 8].

In spite of numerous reports on interactions between the immune system and lymphoma at the intratumoral level, no data have been reported in DLBCL patients on a simple evaluation of the immune status by enumerating peripheral lymphocyte subsets, as currently carried out in viral or iatrogenic acquired immune deficiencies.

In this study, we prospectively determined peripheral TCD4, TCD8, B, natural killer (NK) cell, and monocyte counts upon diagnosis in age-adjusted International Prognostic Index (aaIPI) 2 or 3 DLBCL patients, subsequently treated with anthracyclin-based induction chemotherapy followed by autologous stem-cell transplantation. We found that despite a frequent deficit in all lymphocyte subsets upon diagnosis, only the absolute NK cell count correlated with response to induction treatment and event free survival (EFS), and this independently of aaIPI.

patients and methods

patients and treatment

Patients recruited in this study were <60 years old, diagnosed with a DLBCL confirmed by pathological review, and presenting 2 or 3 adverse prognostic factors of aaIPI (clinical stage III–IV, LDH level above normal, Eastern Cooperative Oncology Group PS 2–4). These patients were included in the LNH98B3 therapeutic clinical trial of the Groupe d’Etude des Lymphomes de l’Adulte (Figure 1). Following informed consent, patients were first randomized for induction treatment. Patients in arm A received four cycles of ACVBP, once every 2 weeks (doxorubicin 75 mg/m² and cyclophosphamide 1200 mg/m² on day (D)1, vindesine 2 mg/m² and bleomycin 10 mg on D1 and D5, oral prednisone 60 mg/m² from D1 to day D5, and intrathecal methotrexate 15 mg on D2). Patients in arm B received one cycle of AC (adriblastin 75 mg/m² on D1, cyclophosphamide 1000 mg/m² on D1 and D2, oral prednisone 60 mg/m² from D1 to D5, and intrathecal methotrexate 15 mg on D2) followed by three cycles of ACE (AC plus etoposide 150 mg/m² on D1, D2, and D3), delivered every 2 weeks. In both arms of induction chemotherapy each cycle was followed by autograft transplantation. Patients in arm A received a high-dose therapy (HDT) regimen with CBV-mitoxantrone conditioning regimen (mitoxantrone 45 mg/m² on D–8, cyclophosphamide 1500 mg/m² on D–7 to D–4, carmustine 300 mg/m² D–4, VP16 250 mg/m² on D–7 to D–4) followed by stem-cell rescue on D0. After evaluation of autotransplantation (D45), responders were once again randomized to receive or not rituximab (375 mg/m²) once a week for 4 weeks. From 10/99 to 05/03 (closing date), 476 patients were enrolled in the clinical trial [9]. Out of the 72 clinical centers which included patients in LNH98B3 trial, 31 participated in the biological study.

evaluation of response and event free survival

Evaluation of response at the end of the inductive cycles (D60) and of the consolidation regimen (D45) allowed to define patients in complete remission (CR), uncertain complete remission (uCR) or PR, according to Cheson criteria [10]. Nonresponders (NR) included patients achieving <50% reduction of the tumor burden, persistence of a progressive disease, treatment alteration or death during the inductive regimen.

EFS was calculated as the duration from the date of inclusion in the LNH98B3 trial to the date of the first event or stopping date (November 2004) or latest follow-up if the latter was anterior to the end point. Events were defined as follows: lymphoma progression during treatment, treatment alteration, lymphoma progression after treatment (PR patients), relapse (CR patients), and death from any cause, without disease progression.

The prognostic impact of peripheral blood cell subsets was assessed comparing responders (CR + uCR + PR) and NR. Patients in PR were merged with responders because this clinical trial involved the same therapeutic regimen for all responders, including PR. Besides, a multinomial logistic analysis comparing CR/uCR patients to PR patients and NR showed no discriminative power of cell counts between PR and CR/uCR groups (data not shown).

immunophenotyping

Absolute counts of peripheral blood main mononuclear subsets, i.e. CD4+, CD8+ T cells, B cells, NK cells, and monocytes, were prospectively carried out in patients upon diagnosis. Samples were analyzed in 17 laboratories, all members of the Groupe d’Etude Immunologique des Leucémies. The procedure was standardized as follows: absolute cell counts were derived directly from the flow cytometry data by using fluorescent beads of calibrated concentration (‘single platform’ technology), as recommended for low cell counts accuracy [11]. Mononuclear cells were defined as follows: lymphocytes were gated according to high-CD45 fluorescence intensity and low side scatter intensity; B cells were defined as CD19+ lymphocytes, CD4 T cells as CD3+CD4+ lymphocytes, CD8 T cells as CD3+CD8+ lymphocytes, NK cells as CD3–CD16+ and/or CD56+ lymphocytes; monocytes were the CD14+ cells among the total leukocytes, i.e. CD45+ elements. Control reagent Immunotrol® (Beckman-Coulter, Nyon, Switzerland) was used as quality control among participant laboratories to ensure homogeneity of measures.

statistical analysis

Comparisons of groups for quantitative data were done by Wilcoxon rank tests. Qualitative data were compared using the chi-square test or the Fischer’s exact test when appropriate. For censored data, EFS curves were estimated by the Kaplan–Meier product-limit method and compared with the log-rank test. Multivariate analyses (Cox regression for censored data, logistic regression for treatment response data) were carried out to evaluate prognostic significance of the potential new predictors adjusted or not on known prognostic factors. In these analyses, log base 2 of lymphocyte subset counts were considered, according to their distribution characteristics (see Figure 2) and model checking analyses [12]. Use of a base 2 in the log transform allows interpreting the hazard ratio (HR) and odds ratio (OR) estimates as the ratio of risks linked to a two-fold increase (or decrease) of the untransformed predictor. P < 0.05 was considered to indicate statistical significance, and confidence intervals were computed with 95% coverage. All calculations were carried out with SAS software, version 8.20 (SAS Institute, Cary, NC) and survival library of R 2.1.1 package [13].

results

patients

From April 1st 2000 to February 1st 2003, 140/476 patients presenting with a DLBCL and included in the LNH98B3 study (Figure 1) were recruited in the biological study. Among them, 13 were primary mediastinal B-cell lymphomas, nine T-cell-rich B-cell lymphomas, and six were associated or developed from small B-cell lymphomas. Clinical characteristics of the patients at diagnosis are listed in Table 1. Two-thirds of these
Figure 1. LNH98B3 protocol schedule. Patients were <60 years old, diagnosed with a diffuse large B-cell lymphoma and 2 or 3 adverse prognostic factors of age-adjusted International Prognostic Index. Patients were first randomized for induction treatment. Patients in arm A received four cycles of doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone once every 2 weeks. Patients in B arm received one cycle of adriblastin and cyclophosphamide followed by three cycles of adriblastin, cyclophosphamide, and etoposide administered every 2 weeks. Patients who achieved at least a partial response after induction treatment received a high-dose therapy with cyclophosphamide, carmustine, and VP16-mitoxantrone conditioning regimen followed by stem-cell rescue at D0. Responders were once again randomized to receive rituximab (375 mg/m²) once a week for 4 weeks or not.

Figure 2. Peripheral lymphocyte subsets and monocyte counts upon diagnosis. Distribution of patients according to counts of CD3⁺CD4⁺ cells (A), CD3⁺CD8⁺ cell (B), CD19⁺ B cells (C), CD3⁻CD16⁺ and/or CD56⁺ natural killer cells (D) monocytes counts using CD14⁺ cells among the total CD45⁺ elements (E).
patients were >40 years old. According to inclusion criteria in the trial, patients presented with high tumor burden, as stated by III–IV Ann Arbor stages and LDH levels above maximal normal values in 98% and 92% of cases, respectively. Forty-three patients (31%) had a score 3 aaIPI. Fifty-five patients (40%) had a lymphopenia with lymphocytes at 700/µl or less.

Sixty-nine patients were randomly enrolled in the ACVBP arm and 71 in the AC/ACE arm. Following induction treatment regimen, 88 of 140 patients (62%) achieved a CR/uCR, 26 (19%) a PR, and 26 (19%) failed to respond. In all, 99 of 114 responders underwent an autograft (49 after ACVBP and 50 after AC/ACE). A second randomization was carried out in 85 patients, and 37 of them received rituximab after HDT (Figure 1). The median follow-up of the analyzed population is 39 months.

The clinical characteristics were similar to those in the overall population of 476 patients enrolled in the LNH98B3 study, according to clinical presentation as well as to clinical outcome [9].

lymphocyte subsets upon diagnosis are quantitatively abnormal in a majority of patients

Out of the 140 patients included in the biological study, 140 had an enumeration of their CD4, CD8, and B-cell subsets, while 136 and 131 had an enumeration of NK cell subsets and monocytes, respectively. As shown in Figure 2, all studied subsets except for monocyte displayed quantitative abnormalities. It should be noted that CD4+ T cells, CD8+ T cells, and NK cells were no longer distributed according to a Gaussian distribution, but showed an increased frequency of low values as well as an increased heterogeneity of the higher values. Compared with reference ranges determined with the same methodology [14], low median cell counts of 337, 211, and 104/µl were evidenced in the CD4+ T cells, CD8+ T cells, and NK cells subsets, respectively (Table 2). Pathological examination of bone marrow was available in 136 patients. The counts of various peripheral blood lymphocyte subsets were similar in patients with (n = 33) or without (n = 104) bone marrow involvement. Similarly, no correlation was found between peripheral blood lymphocyte subsets count and PS or LDH level.

NK cell absolute count upon diagnosis is associated with clinical outcome

Absolute counts of CD4+, CD8+ T cells, B cells, NK cells, and monocytes were correlated with clinical outcome. Two parameters were used to assess the clinical course: (i) response to induction treatment, considering two groups, responders (CR + uCR + PR) and NR and (ii) EFS.

Univariate analysis showed that among subsets only NK cells were associated with disease outcome (Table 3). Higher NK cell counts were significantly associated with response achievement [OR (NK ×2) = 1.81 (1.27, 2.57), P = 0.001]. An average of 159/mm³ was found in responders versus 80/mm³ in NR. Lower NK cell counts upon diagnosis were significantly associated with a shorter EFS [HR (NK/2) = 1.27 (1.06, 1.52) P = 0.01]. No interaction between NK cell counts and response or EFS was observed according to the inductive arm (ACVBP versus AC/ACE). Interestingly, there was no correlation between total lymphocyte counts and induction treatment response or EFS.

The aaIPI used to evaluate the clinical status of patients at diagnosis was also associated in univariate analysis to treatment response [OR (aaIPI 2 versus aaIPI 3) = 2.29 (0.95, 5.45) P = 0.06] and EFS [HR (aaIPI 3 versus aaIPI 2) = 1.95 (1.20, 3.16) P = 0.01].

Therefore to evaluate the respective prognosis value of the NK cell count and the aaIPI, we carried out a multivariate analysis. It showed that NK cell count was associated with treatment response [OR (NK ×2) = 1.77 (1.24, 2.54), P = 0.002]

Table 2. Quantification of circulating mononuclear cells at diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Mean (x/µl³)</th>
<th>Median (x/µl³)</th>
<th>Range (min/max)</th>
<th>Usual values (x/µl³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>823</td>
<td>606</td>
<td>15–3094</td>
<td>1075 (536–1787)</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td>516</td>
<td>337</td>
<td>8–2420</td>
<td>691 (309–1139)</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td>293</td>
<td>209</td>
<td>2–2141</td>
<td>347 (137–827)</td>
</tr>
<tr>
<td>CD3–CD16+CD56+</td>
<td>144</td>
<td>104</td>
<td>5–574</td>
<td>184 (77–427)</td>
</tr>
<tr>
<td>CD19+</td>
<td>133</td>
<td>86</td>
<td>0–1259</td>
<td>170 (72–460)</td>
</tr>
<tr>
<td>CD14+</td>
<td>634</td>
<td>585</td>
<td>45–1764</td>
<td></td>
</tr>
</tbody>
</table>

*By quantification on single platform [22].
Table 3. Univariate analyses of response to treatment and patient event free survival according to peripheral blood mononuclear cell subsets count at diagnosis and aaIPI

<table>
<thead>
<tr>
<th>Induction treatment response</th>
<th>Event free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>CD4+ (n = 140)</td>
<td>1.18 (0.91, 1.54)</td>
</tr>
<tr>
<td>CD8+ (n = 140)</td>
<td>1.30 (0.96, 1.75)</td>
</tr>
<tr>
<td>B &gt;3 (n = 132)</td>
<td>1.16 (0.89, 1.50)</td>
</tr>
<tr>
<td>NK cells (n = 136)</td>
<td>1.81 (1.27, 2.57)</td>
</tr>
<tr>
<td>Mono (n = 131)</td>
<td>1.45 (0.97, 2.17)</td>
</tr>
<tr>
<td>aaIPI (2 versus 3)</td>
<td>2.29 (0.95, 5.45)</td>
</tr>
</tbody>
</table>

and EFS [HR (NK/2) = 1.25 (1.04, 1.50) P = 0.02]

Table 4. Clinical and biological characteristics of patients with low versus normal/high NK cell counts

<table>
<thead>
<tr>
<th></th>
<th>NK ≤80 (n = 71)</th>
<th>NK &gt;80 (n = 85)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (year)</td>
<td>46</td>
<td>48</td>
<td>NS</td>
</tr>
<tr>
<td>Stage 3–4</td>
<td>51 (100%)</td>
<td>82 (96%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. of extranodal sites</td>
<td>32 (74%)</td>
<td>59 (69%)</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate dehydrogenase &gt; NI</td>
<td>46 (90%)</td>
<td>79 (93%)</td>
<td>NS</td>
</tr>
<tr>
<td>ECOG performance status 2–4</td>
<td>21 (41%)</td>
<td>30 (35%)</td>
<td>NS</td>
</tr>
<tr>
<td>Age-adjusted IPI score 3</td>
<td>18 (35%)</td>
<td>21 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>Bone marrow involvement</td>
<td>14 (27%)</td>
<td>17 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>Induction treatment arm ACVBP</td>
<td>25 (49%)</td>
<td>44 (51%)</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR/CR</td>
<td>29 (57%)</td>
<td>58 (68%)</td>
<td>0.001</td>
</tr>
<tr>
<td>PR</td>
<td>5 (10%)</td>
<td>19 (22%)</td>
<td></td>
</tr>
<tr>
<td>Failure</td>
<td>17 (33%)</td>
<td>8 (9%)</td>
<td></td>
</tr>
<tr>
<td>CD3+ cell (median/μl)</td>
<td>514</td>
<td>716</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ cell (median/μl)</td>
<td>296</td>
<td>372</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ cell (median/μl)</td>
<td>194</td>
<td>226</td>
<td>NS</td>
</tr>
<tr>
<td>NK cell (median/μl)</td>
<td>55</td>
<td>170</td>
<td>–</td>
</tr>
<tr>
<td>B cell (median/μl)</td>
<td>72</td>
<td>98</td>
<td>NS</td>
</tr>
<tr>
<td>Monocyte cell (median/μl)</td>
<td>460</td>
<td>598</td>
<td>0.02</td>
</tr>
</tbody>
</table>

aIPI, age-adjusted International Prognostic Index; CI, confidence interval; NS, not significant; NK, natural killer.

discussion

Peripheral blood lymphocyte subsets and monocyte counts were determined at diagnosis in 140 DLBCL patients with 2 to 3 adverse prognostic factors. More than half of the patients presented with lymphocytopenia involving the CD4+, CD8+ T, and NK cell subsets, but only NK cells counts correlated with induction treatment response and EFS.

Sixty one percent of patients under 60 presented severely decreased peripheral T and/or NK lymphocyte counts. To our knowledge, peripheral blood lymphocyte subset counts at diagnosis were never assessed in DLBCL. Lymphopenia is of uncertain origin. Tumor growth has recently been shown to impede functional maturation of NK cell precursors in a mouse model [15]. However, in our patients, no association between any subset deficiency and bone marrow involvement was observed, and lymphocyte subset counts were not associated with tumor burden. This also renders unlikely a potential disturbance of homeostatic signaling by tumor invasion of secondary lymphoid organs.

Global lymphocytopenia is a prognostic factor in Hodgkin’s disease [16]. However, in non-Hodgkin’s lymphomas, data are much less established [17, 18]. Using early death as criteria, either lymphocytopenia <700/μl [19] or leucocyte counts >10 000/μl [20] were reported as pejorative prognostic factors. In our series, using induction treatment response and EFS as prognostic criteria, lymphocytosis did not correlate with disease outcome.

We showed that the absolute NK cell counts are the only blood subset being significantly associated with disease outcome. We cannot exclude that NK cell deficiency is only a surrogate marker of disease severity: for instance, NK cell levels could reveal a stressed condition of the patients [21], which would be the actual cause of the diminished antitumor response. However, this lymphocyte subset was first identified by its ability to kill tumor cells and since NK cells have indeed proven to be important effectors of antitumoral immunity [22]. In our particular setting, Street et al. [23] have shown in mice that NK cells are critical in innate immune surveillance of B-cell lymphomas. Accordingly, the association between a low
numbers could be doubly penalized, with a low NK cell activity.

Hodgkin’s lymphomas, patients presenting with low NK cell activity mediated by NK cells/monocyte macrophages remains a challenge. Antibodies [33]. Since antibody-dependent cellular cytoxicity association of chemotherapy with anti-CD20 monoclonal antibodies designed over the past 3 years included an analysis of the impact of chemotherapy in combination with anti-CD20 monoclonal antibodies on the therapeutic response type in DLBCL.

antitumor cytolytic abilities, could bring some clues to the host response. A better knowledge of the functional subsets of NK cells. Actually, circulating NK cells can be divided in two main subsets, cytolytic ones expressing dimly CD56, contrary to CD56bright cells which do not express cytotoxicity markers [28] and are more engaged in proinflammatory processes [29, 30]. Besides, peripheral NK cells diversely express activating (NCR, NKG2D), coactivating (2B4, DNAM-1), and inhibiting receptors (including NKG2D, NKG2A, NKG2C, CD94, KIR molecules), whose combination might determine the antitumor ability [31, 32]. A better knowledge of these various NK cell subsets, defined according to their antitumor cytolytic abilities, could bring some clues to the host response type in DLBCL.

Moreover, in the field of DLBCL, the great majority of therapeutic trials designed over the last 3 years included an association of chemotherapy with anti-CD20 monoclonal antibodies [33]. Since antibody-dependent cellular cytotoxicity mediated by NK cells/monocyte macrophages remains a privileged mechanism of action of such drugs in non-Hodgkin’s lymphomas, patients presenting with low NK cell numbers could be doubly penalized, with a low NK cell activity and a low rituximab-dependent cytotoxicity. This is under investigation. Also, our results could be useful to avoid overinterpretation of data linking NK cells and mechanism of action of rituximab.

To sum up, we showed that the quantitative NK defect evidenced upon diagnosis in one-third of IPI 2–3 DLBCL patients was associated with induction treatment failure, indicating that NK cell subsets play a role in the response to induction treatment. These results support the potential interest of a treatment targeting the activation of NK cells, in particular, in new therapeutic designs including rituximab.

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references


