Prognostic influence of macrophages in patients with diffuse large B-cell lymphoma: a correlative study from a Nordic phase II trial

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ABSTRACT

The prognostic impact of the tumour microenvironment in diffuse large B-cell lymphoma has not been systematically assessed. We analyzed mRNA and antigen expression of monocytes, macrophages, lymphocytes, dendritic and natural killer cells in pretreatment tumor samples of patients with high-risk diffuse large B-cell lymphoma using gene expression microarray and immunohistochemistry. The patients were treated in a Nordic phase II study with dose-dense chemoimmunotherapy and central nervous system prophylaxis. Of the studied markers for non-malignant inflammatory cells, CD68 expression and CD68+ macrophage counts correlated with favorable outcome. Five-year progression-free survival rates were 83% and 43% for the patients with high and low CD68 mRNA levels, respectively (P=0.007), while overall survival rates were 83% and 64%, respectively (P=ns). The patients with high CD68+ macrophage counts had better 5-year progression-free survival (74% versus 40%; P=0.003) and overall survival (90% versus 60%; P=0.009) than the patients with low macrophage counts. Low CD68+ macrophage count retained its prognostic impact on overall survival with age-adjusted International Prognostic Index [RR=5.0 (95% CI 1.024-19.088); P=0.017]. The findings were validated in three independent cohorts of patients treated with chemoimmunotherapy. In contrast, in patients treated with chemotherapy, high CD68+ macrophage count was associated with poor progression-free survival (40% versus 72%; P=0.021) and overall survival (39% versus 72%; P=0.015). Together, the data suggest that macrophages exhibit a dual, treatment-specific role in diffuse large B-cell lymphoma. For the patients treated with chemoimmunotherapy, high pretreatment CD68 mRNA levels and CD68+ macrophage numbers predict a favorable outcome.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid neoplasm. It is an aggressive disease and only 50% of affected patients can be cured with anthracycline-based CHOP or CHOP-like chemotherapy. Following the addition of rituximab to CHOP, response rates and survival have improved significantly.16 Despite these advances, 20–40% of patients treated with a curative intent experience disease relapses or have primary refractory disease.

The International Prognostic Index (IPI) has so far remained the strongest prognostic factor in DLBCL.17 In general, patients with high IPI scores have a poor prognosis, even if they have received rituximab-containing therapies.1 Nevertheless, the outcome of some patients is comparable to that of low-risk patients, indicating biological diversity within the clinical risk groups.

Gene expression profiling and next-generation sequencing studies have provided seminal biological information to explain the clinical behavior of DLBCL and have also led to the discovery of novel molecular predictors for survival. On the basis of gene expression profiling, DLBCL can be classified into distinct molecular subtypes.9–13 Three major DLBCL entities, showing germinal center B-cell, activated B-cell-like, and primary mediastinal B-cell lymphoma signatures, have been identified. Of these, patients with lymphomas with activated B-cell-like signatures have a shorter survival than patients with either of the other two molecular subtypes.15 In addition, studies based on gene expression profiling have identified the tumor microenvironment and host inflammatory response as defining features in DLBCL.10,15 It is noteworthy that the “stromal-1” signature, which is associated with good outcome after chemoimmunotherapy, includes genes that are typically expressed by components of the extracellular matrix and monocytes.10

At the cellular level, the immune infiltrate in DLBCL comprises macrophages, dendritic cells, mast cells, natural killer cells, innate immune and lymphoid cells including CD4+ T cells (Thelper cells), along with cytotoxic T and non-malignant B cells. Of these, particularly mast cells and tumor-associated macrophages (TAM) have been discovered to have prognostic
impact in DLBCL.\textsuperscript{14-17} Of the macrophages, classically activated M1 type TAM have been described as “good”, acting to prevent the growth of tumor tissue, whereas the alternative M2 type TAM may have an opposite effect promoting angiogenesis and tumor development.\textsuperscript{18-20} Importantly, however, studies in follicular lymphoma have demonstrated that the prognostic significance of the tumor microenvironment and especially macrophages is highly dependent on a given therapy.\textsuperscript{21-25}

In the present study, we investigated how the combination of rituximab with chemotherapy influences non-malignant inflammatory cell-associated clinical outcome in DLBCL. Among all studied markers for macrophages, dendritic, and lymphoid cells, we found that pretreatment gene expression of a macrophage marker CD68 and immunohistochemically defined CD68–TAM content had a positive prognostic impact on the survival of DLBCL patients treated with chemoimmunotherapy, whereas in patients treated without rituximab, CD68–TAM content was associated with a poor outcome.

**Methods**

**Patients and samples**

The screening cohort consisted of prospectively collected DLBCL patients who were less than 65 years old and had primary high-risk (age-adjusted IPI score 2-3) disease. They were treated in the Nordic phase II NLG-LBC-04 protocol with dose-dense chemoinmunotherapy followed by systemic central nervous system prophylaxis.\textsuperscript{24} The patients in this correlative study represent a subset of patients in the main clinical trial and were selected on the basis of DLBCL histology, the availability of fresh frozen tissue for RNA extraction and exon arrays (gene expression cohort; n=38) and formalin-fixed, paraffin-embedded lymphoma tissue containing adequate material for the preparation of tissue microarrays (TMA; immunohistochemistry cohort; n=59), and the patients’ consent to correlative studies. Details of the screening cohort are provided in Table 1, the Online Supplementary Material and Online Supplementary Table S1.

The clinical protocol and sampling were approved by Institutional Review Boards, National Medical Agencies and Ethics Committees in Denmark, Finland, Norway and Sweden, and the trial was registered at ClinicalTrials.gov, number NCT01502982.

To validate the findings, three independent retrospective series of chemoinmunotherapy-treated DLBCL patients were used. In order to confirm gene expression data, we used RNA sequencing data from 92 patients generated by the Cancer Genome Characterization Initiative (CGCI; dbGaP database applied study accession: phs000552.v3.p1)\textsuperscript{25-26} and oligonucleotide-based microarray data from 233 DLBCL patients generated by the Lymphoma/Leukemia Molecular Profiling Project (LLMP; GEO dataset: GSE10846).\textsuperscript{27} Both cohorts are subsets of the original study populations treated with a R-CHOP-like regimen based on the availability of complete expression data and clinical information (Online Supplementary Table S2).

In order to confirm the immunohistochemical data, an independent population-based series of 72 primary DLBCL patients treated with chemoimmunotherapy at the Helsinki University Central Hospital between 2001 and 2006 was used (Table 2). In addition, 50 DLBCL patients treated with chemotherapy in Helsinki before rituximab was adopted into clinical routine, and the LLMP pre-rituximab cohort (n=181) treated with CHOP\textsuperscript{18} served as pre-rituximab control groups. Details of the validation cohorts are provided in the Online Supplementary Material.

**Gene expression**

Gene expression levels of CD68, CD163, and C-C motif chemokine ligand 18 (CCL18) were determined from the exon array-based data set of 38 pre-treatment lymphoma samples (Affymetrix Human Exon 1.0 ST arrays) from the patients treated in the Nordic phase II NLG-LBC-04 protocol,\textsuperscript{27} and from the data set of ten pairs of lymphoma samples collected before and a day after the first course of R-CHOP. Hybridization protocols and raw expression microarray data are available at the ArrayExpress archive (http://www.ebi.ac.uk/microarray-as/aID: E-MEXP-3488 and ID: E-MTAB-2471).

**Immunohistochemistry**

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissue sections on TMA slides or whole tissue sections (independent validation cohorts) with antibodies against CD68, CD163, CCL18, CD3, CD4, CD8, CD14, CD21, CD57 and GCT1 according to the manufacturer’s instructions. Detailed information on the antibodies and scoring of the staining are described in the Online Supplementary Material.

**Statistical analyses**

Data were analyzed using PASW Statistics 18.0 (SPSS, Inc.) and are described in detail in the Online Supplementary Material. Probability values below 0.05 are considered statistically significant and all P values are two-tailed.

**Table 1. Baseline characteristics and outcome of the original study population, and exon array and TMA cohorts from the Nordic phase II study.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Clinical trial\textsuperscript{a}</th>
<th>Gene expression (exon array)</th>
<th>Immunohistochemistry (TMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>143</td>
<td>38</td>
<td>59</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>54 (18-65)</td>
<td>53 (20-64)</td>
<td>53 (18-65)</td>
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<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>104 (73)</td>
<td>29 (76)</td>
<td>44 (75)</td>
</tr>
<tr>
<td>≥60 years</td>
<td>39 (27)</td>
<td>9 (24)</td>
<td>15 (25)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>92 (64)</td>
<td>24 (63)</td>
<td>41 (69)</td>
</tr>
<tr>
<td>Female</td>
<td>51 (36)</td>
<td>14 (37)</td>
<td>18 (31)</td>
</tr>
<tr>
<td>Age-adjusted IPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>104 (73)</td>
<td>27 (71)</td>
<td>42 (71)</td>
</tr>
<tr>
<td>3</td>
<td>39 (27)</td>
<td>11 (29)</td>
<td>17 (29)</td>
</tr>
<tr>
<td>DLBCL molecular subgroup</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCB</td>
<td>72 (50)</td>
<td>24 (63)</td>
<td>32 (54)</td>
</tr>
<tr>
<td>Non-GCB</td>
<td>32 (22)</td>
<td>9 (24)</td>
<td>21 (36)</td>
</tr>
<tr>
<td>PMBL</td>
<td>7 (5)</td>
<td>2 (5)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>32 (22)</td>
<td>3 (8)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Relapses</td>
<td>43</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Deaths</td>
<td>34</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Lymphoma-specific</td>
<td>25</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>5-year PFS</td>
<td>70%</td>
<td>72%</td>
<td>74%</td>
</tr>
<tr>
<td>5-year OS</td>
<td>78%</td>
<td>76%</td>
<td>84%</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cases with follicular lymphomas have been excluded; GCB: germinal center B-cell-like; PMBL: primary mediastinal B-cell lymphoma; PFS: progression-free survival; OS: overall survival.
Results

Characteristics of the patients and diseases
The clinical characteristics of the prospective cohorts of
patients who received treatment in the Nordic phase II
study are listed in Table 1. There were no major differ-
ces in the baseline characteristics or outcomes between
the cases originally included in the trial and the cases
available for the correlative studies, implying that the
cases were representative of the entire clinical trial. The
median follow up was 65 months for both the gene
expression and TMA cohorts. In the gene expression
cohort (n=38), nine patients had relapsed and nine died.
Two of the deaths were not lymphoma-related, including
one toxic death and one suicide. Five-year progression-
free survival (PFS) and overall survival (OS) rates were
72% and 76%, respectively. In the TMA cohort (n=59),
there were 16 relapses and 11 deaths. One of the deaths
was due to secondary cancer. Five-year PFS and OS rates
were 72% and 85%, respectively.

Gene expression data
We utilized the exon array database to address whether
there was a correlation between survival and gene expres-
sion of CD68, CD163, and CCL18 in the lymphoma tissue.
Of these, CD68 encodes a pan-macrophage marker
expressed on both classically activated M1 type and alter-
native M2 type macrophages, whereas CD163 expression
is more specific for the latter subtype. Likewise, CCL18
expression has been recognized as a marker for M2 type
macrophages. The baseline characteristics of the array
cohort are shown in Table 1. CD68 gene expression was
found to correlate with CD163 (r=0.574, p<0.001) and
CCL18 (r=0.426, p=0.008) mRNA levels. When the associ-
ation between gene expression and survival was analyzed,
high CD68 mRNA levels were found to have a favorable
impact on PFS (p=0.016) although not on OS (p=ns), and no
associations were found between CD163 or CCL18
expression and survival parameters. According to Kaplan-
Meier analysis using an optimal cutoff level of 57%, the
5-year PFS rate for the patients with high CD68 mRNA
levels was 83% as compared with 43% for the patients with
low CD68 expression (p=0.009; Figure 1A). The correspon-
ding 5-year OS rates were 53% and 65% (p=ns; Figure 1B).
When clinical characteristics of the patients were com-
pared according to CD68 expression, no significant differ-
ce in age, gender, age-adjusted IPI scores, or molecular
subtype were observed between the subgroups (Online
Supplementary Table S1).
In order to find support for our CD68 expression data,
we analyzed the prognostic significance of CD68 gene
expression in the CGCI and LLMPP data sets. The char-
acteristics of the patients in these cohorts and their diseases
are described in the Online Supplementary Material (Online
Supplementary Table S2). In the CGCI cohort, high CD68
gene expression predicted favorable OS (p=0.033). When
the optimal cutoff level of 35% was used to discriminate
the outcomes between the low and high CD68 subgroups,
the 3-year OS rate of the patients with high CD68 mRNA
levels was 86% compared with 67% for those with low
levels (p=0.040; Figure 1C). When the originally defined
cutoff level was used (37%), the difference between the low
and high CD68 subgroups was of borderline signifi-
cance (p=0.071). In the LLMPP data set with the optimal
cutoff level of 24%, 5-year OS rates were 72% and 58%
for the patients with high and low CD68 mRNA levels,
respectively (p=ns; Figure 1D).

Association of CD68 tumor-associated macrophage
content with survival
Next, we analyzed the prognostic impact of CD68 TAM
content on survival in the Nordic phase II study population.
Tumor tissue for TMA analysis and immunohistochemistry
was available for 59 patients (Table 1). Number of CD68 TAM
correlated with CD68 gene expression (r=0.584,
P=0.009) and CD163 TAM counts (r=0.780, P<0.001).
Likewise, CD163 TAM counts correlated with CD163
(r=0.597, P=0.005) gene expression. Furthermore, there was a good concordance for CD68 TAM analyses performed in two laboratories
(r=0.770, P<0.001).

The median level of CD68 TAM/high power field (hpf)
was 37 (range, 5-95). The cutoff level of 26 TAM/hpf,
corresponding to 17%, was found to discriminate best between
subgroups with different outcomes. According to Kaplan-
Meier estimates, the patients with high CD68 TAM counts
had a 5-year PFS of 74% in comparison to 40% for those
with lower CD68 TAM counts (P=0.003; Figure 2A), and a
better 5-year OS (90% versus 60%, P=0.009; Figure 2B). In
multivariate analysis with age-adjusted IPI, low CD68 TAM
count retained its adverse prognostic value on OS (CD68-
TAM, RR=5.037 (95% CI 1.329-19.088), P=0.017; IPI, RR=3.981 (95% CI 1.024-14.578), P=0.046). No differences
were observed in stage, IPI scores or molecular subgroups.

Table 2. Baseline characteristics according to CD68 TAM content in the chemoimmunotherapy validation cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>CD68 TAM Low</th>
<th>CD68 TAM High</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>72</td>
<td>55</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Median age (range)</td>
<td>64 (20-80)</td>
<td>65 (20-80)</td>
<td>63 (42-77)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>28 (39)</td>
<td>21 (38)</td>
<td>7 (41)</td>
<td>1.000</td>
</tr>
<tr>
<td>≥60 years</td>
<td>44 (61)</td>
<td>34 (62)</td>
<td>10 (59)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41 (57)</td>
<td>29 (53)</td>
<td>12 (71)</td>
<td>0.265</td>
</tr>
<tr>
<td>Female</td>
<td>31 (45)</td>
<td>26 (47)</td>
<td>5 (29)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>34 (47)</td>
<td>26 (47)</td>
<td>8 (47)</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
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<td>28 (51)</td>
<td>9 (53)</td>
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<tr>
<td>Missing</td>
<td>1 (1)</td>
<td>1 (2)</td>
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<tr>
<td>IPI score</td>
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<tr>
<td>0-2</td>
<td>49 (68)</td>
<td>38 (69)</td>
<td>11 (65)</td>
<td>0.771</td>
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<tr>
<td>3-5</td>
<td>23 (32)</td>
<td>17 (31)</td>
<td>6 (35)</td>
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<tr>
<td>Molecular subgroup</td>
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<td></td>
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<tr>
<td>GCB</td>
<td>18</td>
<td>14 (25)</td>
<td>4 (24)</td>
<td></td>
</tr>
<tr>
<td>Non-GCB</td>
<td>27</td>
<td>22 (40)</td>
<td>5 (29)</td>
<td>1.000</td>
</tr>
<tr>
<td>Missing</td>
<td>27</td>
<td>19 (35)</td>
<td>8 (47)</td>
<td></td>
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<tr>
<td>Relapses</td>
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<td>17</td>
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<td>0.031</td>
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<td>Deaths</td>
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<td>Other</td>
<td>14</td>
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<td></td>
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<tr>
<td>Missing</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

GCB: germinal center B-cell like.
between the low and high CD68+ TAM groups (Online Supplementary Table S1).

To determine the prognostic impact of other cells in the reactive microenvironment on survival, mRNA levels of the pan T-lymphocyte marker CD3 (chains e, d and g), T-helper cell antigen CD4, cytotoxic T-cell antigen CD8 (chains a and b), monocyte antigen CD14, follicular dendritic cell marker CD21 and natural killer-cell marker CD57, as well as germinal center B-cell marker GCET1 were analyzed. Furthermore, the samples were immunostained and quantified for CD163, CCL18, CD3, CD4, CD8, CD14, CD21, CD57 and GCET1 positivity. For all markers, the gene expression levels correlated with the corresponding cell counts, and CD14+ cell counts correlated with CD68+ TAM. However, neither gene expression levels nor any of the cell counts were associated with survival.

**Association of tumor-associated macrophages with survival in an independent series of patients with diffuse large B-cell lymphoma**

To validate the positive prognostic value of high CD68+ TAM content in the rituximab era, we used an independent population-based set of 72 DLBCL patients treated with chemoimmunotherapy. The baseline characteristics of the validation cohort are listed in Table 2. Instead of a TMA-based analyses, immunohistochemical staining was performed on individual whole tissue sections. The relations between CD68+ TAM and baseline characteristics are shown in Table 2. In this cohort, the median level for CD68+ TAM/hpf was 33 (range, 13-67). The cutoff level of 43 CD68+ TAM/hpf corresponding to 76% was found to discriminate best between subgroups with different outcomes. When this cutoff level was used, no differences were observed in age, stage, IPI scores or molecular subgroups between patients in the low and high CD68+ TAM groups.

The clinical outcomes according to treatment and CD68+ TAM content in the validation cohort are shown in Figure 3. According to Kaplan Meier analysis, 5-year PFS rates were 88% and 65% for the patients with high and low numbers of CD68+ TAM, respectively (P=0.05; Figure 3A). The corresponding OS rates were 88% and 72%, respectively (P=ns; Figure 3B). The risk of relapse was 3.7-fold higher for the patients with low CD68+ TAM counts (95% CI 0.903-16.462, P=0.068) and 2.5-fold higher for the patients with high IPI scores (CI 95% 1.104-5.492, P=0.022). In multivariate analyses with IPI, the negative prognostic impact of low TAM content on OS was of borderline significance (RR 4.1, 95% CI 0.948-17.309, P=0.089; for IPI, RR 2.6, 95% CI 1.149-5.722, P=0.021).

**Response of tumor-associated macrophages to treatment**

To evaluate the impact of therapy on TAM, we analyzed the expression of macrophage markers and the number of TAM from ten DLBCL pairs of samples collected before the treatment and 1 day after the first chemoimmunotherapy infusion had ended. Comparison of pre- and post-treatment tissue samples in this small cohort of patients showed an increase in mRNA levels of CD68 (P=0.052), CD163 (P=0.023) and CCL18 (P=0.042) genes in response to therapy (Figure 4A). Consistent with the gene expression data, a significant increase in the number of CD68+ TAM (53 versus 68, P=0.023) (Figure 4B), and a non-significant increase in the
CD163⁺ TAM counts (67 versus 77) was also observed. CD3 (CD3e, P=0.003; CD3d, P=0.010; CD3g, P=0.013), CD4 (P=0.021), and CD8 (CD8a, P=0.009) mRNA levels also increased in response to therapy, while CD20 and CD21 levels did not show significant changes.

**Association of tumor-associated macrophages with survival in the pre-rituximab era**

Finally, we evaluated the prognostic impact of TAM on the outcome of 50 DLBCL patients who received therapy before rituximab was routinely available (pre-rituximab era). The characteristics of the patients in this pre-rituximab cohort are shown in Online Supplementary Table S3. Thirty patients received high-dose therapy and autologous stem cell transplantation as consolidation after their first-line therapy, and 20 as salvage therapy for relapsed disease. The median number of CD68⁺ TAM/hpf was 58 (range, 19-83). No differences were observed in baseline characteristics between patients in the high and low CD68⁺ TAM subgroups (Online Supplementary Table S3).

According to Kaplan-Meier estimates (Online Supplementary Figure S1), the patients with low CD68⁺ TAM content had better 5-year PFS and OS rates when compared to patients with high CD68⁺ TAM counts (72% versus 40%, P=0.021 for PFS and 72% versus 39%, P=0.015 for OS). When the patients were divided into two groups according to the time of autologous transplantation, the patients treated with high-dose therapy as salvage therapy and with low CD68⁺ TAM counts at diagnosis had significantly better OS than the ones with high counts (70% versus 13%, P=0.020). A non-significant difference was also observed in the patients who received the treatment frontline (80% versus 47%, P=0.085). The results confirm previous findings in chemotherapy-treated patients. 15,16

To complement the data on CD68⁺ TAM, we analyzed CD68 gene expression in relation to OS from the LLMPP CHOP cohort. With the cutoff level of 19%, 5-year OS rates were 45% and 55% for the patients with high and low CD68 mRNA levels (P=ns; Online Supplementary Figure S2). Together, the data suggest that addition of rituximab to chemotherapy reverses the negative prognostic impact of high CD68⁺ TAM content to favorable.

**Discussion**

Our aim was to determine how the combination of rituximab with chemotherapy influences tumor infiltrating inflammatory cell-associated survival in DLBCL. The results from a prospectively collected screening cohort of patients treated homogenously in the Nordic phase II study showed that high CD68 gene expression and high number of CD68⁺ TAM were associated with favorable PFS and OS in DLBCL patients treated with chemoimmunotherapy. Furthermore,
CD68+ TAM content was identified as an independent risk factor for OS. In comparison, no correlation between CD163, CCL18, CD14, CD21, CD3, CD4, CD8 and CD57 expression or corresponding antigen-positive cells and outcome was found. The results on CD68 expression and CD68+ TAM counts were validated in three larger independent cohorts. In addition, we demonstrated an inflammatory response including an increased TAM content in response to chemoimmunotherapy.

Macrophages are divided into at least M1 and M2 subtypes, which express different levels of cell surface markers, adhesion molecules, scavenger receptors, chemokines, cytokines, and receptors, and show different effector functions. Studies on solid tumors have shown that TAM display a M2 type phenotype, and that TAM content often correlates with poor survival. We observed that in DLBCL patients treated with chemotherapy alone, high CD68+ TAM content was associated with a poor prognosis. However, in the case of lymphomas treated with a rituximab-containing regimen, the effect of CD68+ macrophages in the lymphoma tissue was opposite. The data allow the speculation that TAM can switch from a tumor-promoting to a tumor-inhibiting function in response to rituximab.

Our analyses showed a correlation of CD68 mRNA levels and immunohistochemically defined CD68+ TAM counts with survival, whereas no association between the expression of M2 type macrophage markers CD163 or CCL18 and survival was found. Of these antigens CD163 is a hemoglobin scavenger receptor playing a major role in dampening the inflammatory response and in scavenging components of damaged cells. CCL18 expression is known to contribute to the active recruitment of lymphocytes and immature dendritic cells under inflammatory and pathological conditions. Interestingly, CCL18 has also been shown to be able to stimulate monocytes to mature into macrophages. Although the terms M1 and M2 macrophages are simplifications of reality, they have been used to explain the opposing functions of different macrophage subsets. A large proportion of M1 type TAM in the total macrophage content may provide better tumor control, since the overall balance in the tumor microenvironment shifts to the anti-tumor response. If M2 type macrophages predominate, the balance may, instead, shift to a pro-tumor microenvironment. Our findings in the chemoimmunotherapy-treated patients may, therefore, reflect the anti-tumor effects of M1 type TAM dominating over the pro-tumor effect of the M2 type TAM, whereas in the chemotherapy-treated cohort the effect is opposite.

In our patients, both CD68 mRNA levels and CD68+ TAM content predicted outcome. Similar results were also recently reported at the protein level by others. The findings at multiple levels are important because the optimal cutoff levels best discriminating the low and high subgroups varied between different cohorts. Several explanations for this variability are possible. It is likely that true microenvironment-related differences exist between different populations of patients. However, it is also conceivable that methodological differences, especially in specimen preparation (TMA versus whole sections), and intratumoral heterogeneity influence scoring results. Furthermore, we recognize the limitations of small cohorts of patients. Thus, data from different series should be compared with special caution. It is also important to emphasize that we do not consider certain cutoff points to be biologically significant, but rather think that CD68 expression and CD68+ TAM levels form a continuum with increased numbers of TAM correlating with improved survival in patients treated with chemoimmunotherapy.

At present it remains unclear how TAM could switch from a tumor-promoting to tumor-suppressing function when rituximab is combined with chemotherapy. Nevertheless, macrophages have been implicated as crucial players in the mechanisms of actions of CD20 antibodies including rituximab. They mediate antibody-dependent cellular phagocytosis, and are also involved in antibody-dependent cellular cytotoxicity. Interestingly, preclinical data demonstrate that M2 type macrophages phagocytose rituximab-opsonized lymphoma cells more efficiently than do M1 type cells. Our findings that macrophage markers and content increase in tumor tissue in response to a rituximab-containing regimen enable speculation that a favorable outcome could be a consequence of more efficient macrophage-mediated phagocytosis of lymphoma cells. Macrophages may also secrete cytolytic factors or release cytokines, thereby recruiting other effector cells to amplify the inflammatory response. Conversely, lymphoma cells can secrete cytokines, including interleukin-10, which favor alternative activation of macrophages to M2 type cells. Considering that tissue macrophages are critical for B-cell depletion after anti-CD20 therapy, it is possible.
that there is an interrelationship between TAM content and efficacy of rituximab. Together the data support a hypothesis that macrophages mediate the therapeutic activity of rituximab treatment.9,15-40

An additional aim of this study was to explore the effect of chemoimmunotherapy on the tumor microenvironment. The analysis has limitations due to the small number of evaluable patients and lack of control group treated without rituximab; however, when pre-treatment and 1-day post-treatment samples were compared, an increase in gene expression of macrophage markers and TAM in response to chemoimmunotherapy was found. This observation is in line with in vitro findings showing the immune-stimulatory effect of rituximab on macrophages.9,37 Alternatively, the finding may reflect an association of increased numbers of macrophages with a tissue repair process during chemotherapy-induced cell death. A more detailed comparison of the composition of the tumor microenvironment at diagnosis, during treatment and at relapse will be instrumental in identifying the cellular niche which is either promoting drug-induced lymphoma cell death or alternatively protecting lymphoma cells from cytotoxicity.

Macrophage-mediated lymphoma depletion during chemoimmunotherapy may have clinical implications. For example, it may be possible to stimulate macrophage activity further in the tumor tissue with the use of granulocyte-macrophage or macrophage colony-stimulating factors (GM-CSF or M-CSF).37,39 Administration of GM-CSF or M-CSF activates numerous immune cells, notably granulocytes and monocytes, which express Fcγ receptors and are involved in rituximab-mediated antibody-dependent cellular cytotoxicity and phagocytosis.34,41 Furthermore, GM-CSF can upregulate CD20 expression on lymphoma cells.39 The concept of GM-CSF-induced immune priming has been successfully used in combination with rituximab monotherapy in patients with relapsed or progressive follicular lymphoma,40 and recently also tested with R-CHOP in patients with primary DLBCL.41,42 Another novel and promising immunotherapeutic approach is to augment rituximab-mediated anti-body-dependent cellular phagocytosis by combining rituximab with another antibody or a peptide, which inhibits the CD47-mediated antiphagocytic signaling pathway that the cancer cells use to inhibit macrophage-mediated destruction.43-46

Unlike CD68, TAM, the rest of the cells present in the reactive microenvironment had no impact on survival. Our results for the T-cell markers CD8 and CD4 differ from those in a recently published study, in which high levels of CD8+ and CD4+ T cells were associated with favorable prognosis.47 Due to major differences in the study populations, treatments and methodologies used for cell quantification (immunohistochemistry versus flow cytometry), the results from these studies are not comparable.

In conclusion, we have shown that TAM exhibit a dual treatment-dependent role in the pathogenesis of DLBCL. While high TAM counts are associated with poor outcome in patients treated with chemotherapy, this adverse prognostic impact is reversed when rituximab is combined with chemotherapy. Similar functional plasticity of TAM has previously been shown to occur in follicular lymphoma.23,24 The strengths of our study are a prospectively collected and homogenously treated study population, the availability of gene expression and immunohistochemical data from the same cohort of patients, and the possibility of validating the results in three independent cohorts of DLBCL patients. The limitations of the study include relatively small sample sizes, disparate study populations and different specimen preparations (TMA versus whole sections). Furthermore, we recognize that the TMA design does not optimally reflect the entire distribution of tumor infiltrating inflammatory cells because of regional variation in their localization. Nevertheless, our findings establish a macrophage marker, CD68, to be important in predicting the survival of patients with DLBCL and warrant its evaluation in prospective, clinical trials of DLBCL.

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Authorship and Disclosures

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