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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 tumor 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.[1-6] 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.[7,8] In general, patients with high IPI scores have a poor prognosis, even if they have received rituximab-containing therapies.[8] 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.[10] In addition, studies based on gene expression profiling have identified the tumor microenvironment and host inflammatory response as defining features in DLBCL.[10,13] 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 (T-helper 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

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impact in DLBCL.[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.[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.[21-23]

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 chemoimmunotherapy followed by systemic central nervous system prophylaxis.[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 chemoimmunotherapy-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: phs000532.v3.p1)[25,26] and oligonucleotide-based microarray data from 233 DLBCL patients generated by the Lymphoma/Leukemia Molecular Profiling Project (LLMPP; GEO dataset: GSE10846).[10] 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 LLMPP pre-rituximab cohort (n=181) treated with CHOP[10] 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,[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/ae/ID: 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 GCET1 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.

Characteristic	Clinical trial[1]	Gene expression (exon array)	Immunohistochemistry (TMA)
Number of patients	143	38	59
Median age (range)	54 (18-65)	53 (20-64)	53 (18-65)
Age			
<60 years	104 (73)	29 (76)	44 (75)
≥60 years	39 (27)	9 (24)	15 (25)
Gender			
Male	92 (64)	24 (63)	41 (69)
Female	51 (36)	14 (37)	18 (31)
Age-adjusted IPI			
2	104 (73)	27 (71)	42 (71)
3	39 (27)	11 (29)	17 (29)
DLBCL molecular subgroup			
GCB	72 (50)	24 (63)	32 (54)
Non-GCB	32 (22)	9 (24)	21 (36)
PMBL	7 (5)	2 (5)	3 (5)
Unknown	32 (22)	3 (8)	3 (5)
Relapses	43	10	16
Deaths	34	9	11
Lymphoma-specific	25	5	9
Other	9	4	3
5-year PFS	70%	72%	74%
5-year OS	78%	76%	84%

[1]*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[24] are listed in Table 1. There were no major differences 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[27] to address whether there was a correlation between survival and gene expression 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 alternative 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.[28] The baseline characteristics of the array cohort are shown in Table 1. *CD68* gene expression was found to correlate with *CD163* (r_s=0.574, *P*<0.001) and *CCL18* (r_s=0.426, *P*=0.008) mRNA levels. When the association 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 37%,[29] 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 corresponding 5-year OS rates were 83% and 63% (*P*=ns; Figure 1B). When clinical characteristics of the patients were compared according to *CD68* expression, no significant differences in age, gender, age-adjusted aIPI 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[25,26] and LLMPP[10] data sets. The characteristics 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 significance (*P*=0.071). In the LLMPP data set with the optimal cutoff level of 23%, 5-year OS rates were 72% and 58% for the patients with high and low *CD68* mRNA levels, respectively (*P*=ns; Figure ID).

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_s=0.584, *P*=0.009) and CD163$^+$ TAM counts (r_s=0.780, *P*=0.001). Likewise, CD163$^+$ TAM counts correlated with *CD163* (rs=0.857, *P*<0.001) and *CD68* (r_s=0.597, *P*=0.005) gene expression. Furthermore, there was a good concordance for CD68$^+$ TAM analyses performed in two laboratories (r_s=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.

Characteristic		CD68$^+$ TAM		
	All	Low	High	*P*
Number of patients	72	55	17	
Median age (range)	64 (20-80)	65 (20-80)	63 (42-77)	
Age				
<60 years	28 (39)	21 (38)	7 (41)	1.000
≥60 years	44 (61)	34 (62)	10 (59)	
Gender				
Male	41 (57)	29 (53)	12 (71)	
Female	31 (43)	26 (47)	5 (29)	0.265
Stage				
I-II	34 (47)	26(47)	8 (47)	
III-IV	37 (52)	28 (51)	9 (53)	1.000
Missing	1 (1)	1 (2)		
IPI score				
0-2	49 (68)	38 (69)	11 (65)	
3-5	23 (32)	17 (31)	6 (35)	0.771
Molecular subgroup				
GCB	18	14 (25)	4 (24)	
Non-GCB	27	22 (40)	5 (29)	1.000
Missing	27	19 (35)	8 (47)	
Relapses	18	17	1	0.031
Deaths				
Lymphoma-specific	20	18	2	0.125
Other	14	13	1	
	6	5	1	

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*=0.050; 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*=0.028). In multivariate analyses with IPI, the negative prognostic impact of low TAM content on PFS was of borderline significance (RR 4.1, 95% CI 0.948-17.309, *P*=0.059; 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

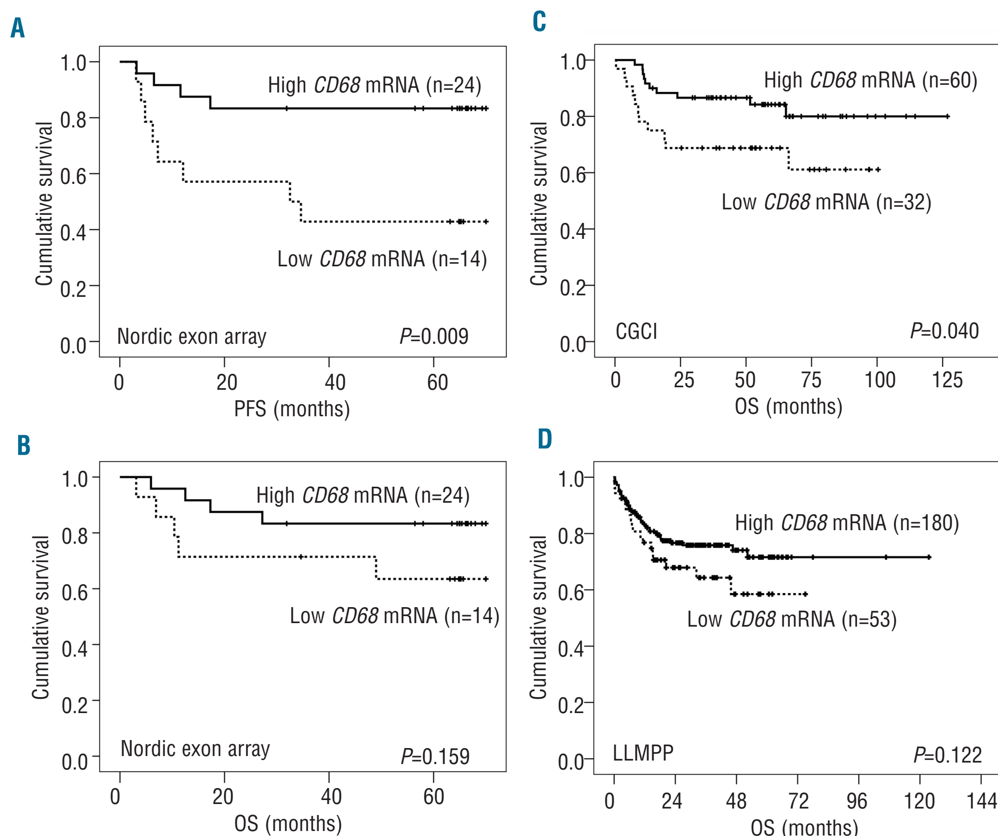

Figure 1. Survival according to *CD68* gene expression in different chemoimmunotherapy cohorts. PFS (A) and OS (B) rates according to low and high *CD68* gene expression in the Nordic phase II exon array cohort. (C) OS according to *CD68* gene expression in the CGCI cohort. (D) OS according to *CD68* gene expression in the LLMPP cohort.

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*=0.083). 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,

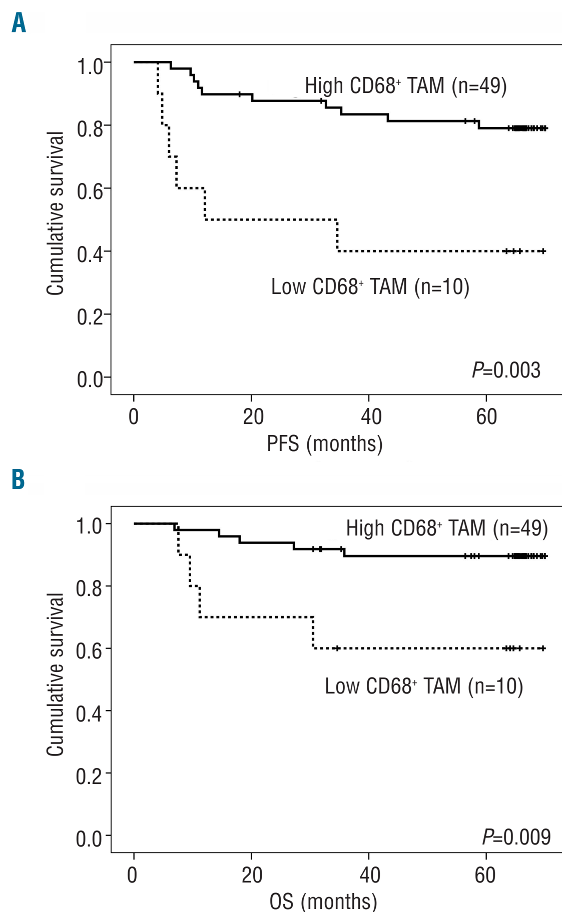

Figure 2. PFS (A) and OS (B) according to high and low CD68+ TAM in the Nordic phase II TMA cohort.

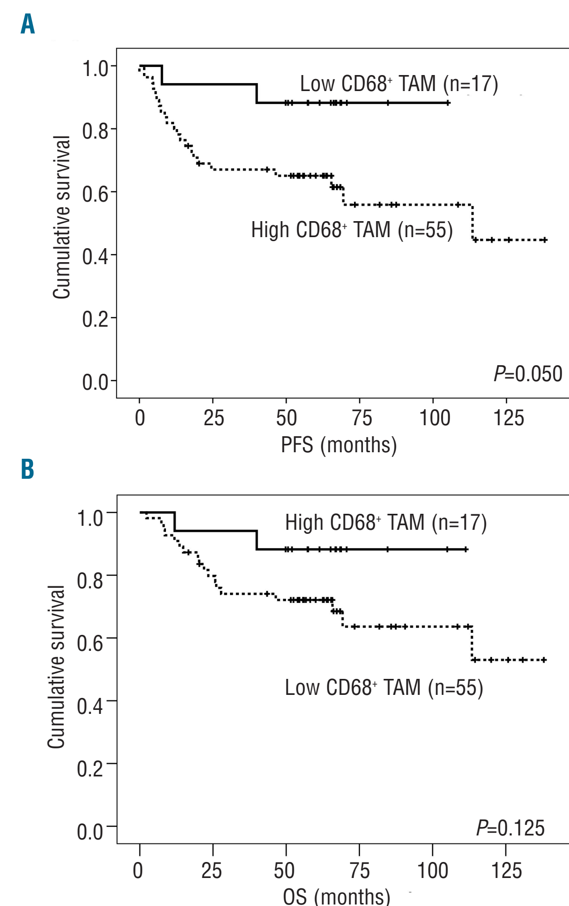

Figure 3. PFS (A) and OS (B) according to CD68+ TAM in the chemoimmunotherapy validation cohort.

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.[19,20] Studies on solid tumors have shown that TAM display a M2 type phenotype,[30,31] 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.[32] CCL18 expression is known to contribute to the active recruitment of lymphocytes and immature dendritic cells under inflammatory and pathological conditions.[28] Interestingly, CCL18 has also been shown to be able to stimulate monocytes to mature into macrophages.[33] 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.

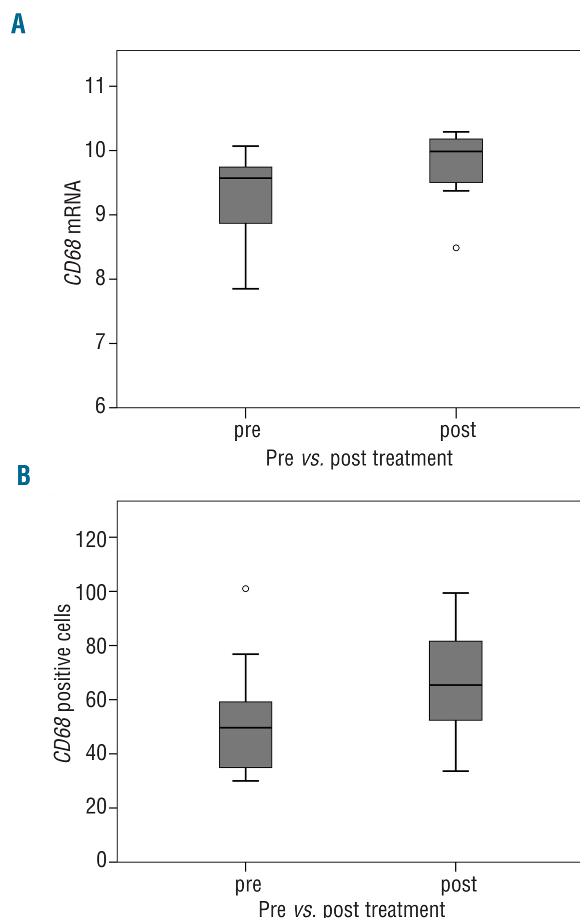

Figure 4. (A) **Expression of *CD68* mRNA before and after the first R-CHOP course.** (B) **Number of CD68+ TAM before and after the first R-CHOP course.**

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.[17] 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.[34-36] 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.[37] 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,[34,35,38] 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.[36,38-40]

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.[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.[39,41] Furthermore, GM-CSF can upregulate CD20 expression on lymphoma cells.[42] 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,[43] and recently also tested with R-CHOP in patients with primary DLBCL.[44,45] Another novel and promising immunotherapeutic approach is to augment rituximab-mediated antibody-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.[40,46]

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 CD3 and CD4 differ from those in a recently published study, in which high levels of CD3$^+$ 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 inversed when rituximab is combined with chemotherapy. Similar functional plasticity of TAM has previously been shown to occur in follicular lymphoma.[21,23] 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 preparation (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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