Med Oncol (2012) 29:3522–3528
DOI 10.1007/s12032-012-0244-6

ORIGINAL PAPER

High numbers of tumor-associated macrophages correlate with poor prognosis in patients with mature T- and natural killer cell lymphomas

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Received: 25 February 2012 / Accepted: 17 April 2012 / Published online: 5 May 2012

Abstract Various studies on lymphoma microenvironment have demonstrated the prognostic impact of tumor-associated macrophages (TAMs) in patients with B-cell lymphoma. Little is known about the correlation between TAMs and treatment outcome in mature T- and natural killer (NK) cell lymphomas. We analyzed the prognostic relevance of CD68+ TAMs by immunohistochemical analysis in 64 Chinese patients with mature T- and NK-cell lymphomas. Higher number of infiltrated TAMs was significantly related to B symptoms and extranodal involvement ($p < 0.05$). The TAMs content did not differ significantly between pathological subtypes. Using the mean value of TAMs per high-power field (hpf) as the cutoff point (87/hpf), 36 cases (56.2 %) were categorized as low level of TAMs content and 28 cases (43.8%) as high level. Patients with high level of TAMs content had a worse 5-year overall survival compared to those with low level (28.1 vs. 44.3 %, $p = 0.039$). In multivariate analysis, TAMs content remained an independent biological variable for survival distinct from the International Prognostic Index (Cox multivariate model, $p = 0.009$). High TAMs content indicated an adverse overall outcome in mature T- and NK-cell lymphomas. Our results show that expression of stromal TAMs may become a useful marker for prognosis of mature T- and NK-cell lymphomas.

Keywords Tumor-associated macrophages · Mature T- and NK-cell lymphomas · Tumor microenvironment · Prognosis · CD68

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Introduction

It is becoming more and more evident that the interaction between the inflammatory microenvironment and tumor cells plays an indispensable role in the development and progression of tumor [1, 2]. Tumor-associated macrophages (TAMs), which form a pivotal component of the inflammatory infiltrate in virtually all types of malignancy, exhibit a distinct phenotype and express pro-tumoral functions [3–5]. Many observations indicate that TAMs may produce a vast diversity of growth factors, proteolytic enzymes, pro-angiogenic cytokines, and inflammatory mediators, which not only directly stimulate the growth of tumor cells and/or facilitate tumor metastatic invasion but also induce immune suppression of host defenses against tumor [3, 4]. The pro-tumoral role of TAMs is supported by many clinical cancer studies including breast, prostate, endometrial, and bladder cancers, which found a correlation between the high macrophage content and poor prognosis [5]. Various studies have also drawn attention to the prognostic effect of TAMs in patients with B-cell non-Hodgkin lymphoma (NHL). In follicular lymphoma (FL), the biological and clinical behavior is determined particularly by the tumor microenvironment. High amounts of CD68+ TAMs have been shown to correlate with inferior outcome [6–9], although it appears to be dependent on the specific treatment protocol used [10, 11]. In diffuse large

B-cell lymphoma (DLBCL), results of immunohistochemical studies on TAMs seemed contradictory, but most of them showed a significant inverse correlation between the number of CD68+ cells and overall survival [12–14]. However, little is known about the prognostic significance of TAMs in the stroma of mature T- and natural killer (NK)-cell lymphomas.

Mature T- and NK-cell lymphomas are a histologically and clinically heterogeneous group of lymphoid malignancies derived from mature or post-thymic T cells and NK cells, accounting for 15–20 % of all NHL in Asia [15, 16]. Unlike B-cell NHL, most cases of mature T- and NK-cell lymphomas are clinically aggressive tumors that cannot be classified according to the normal stages of T-cell differentiation [17]. With the exception of anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALCL), patients with mature T- and NK-cell lymphomas usually have a dismal prognosis with the 5-year overall survival of less than 50 % after standard CHOP-type chemotherapy [18].

In this study, we measured by immunohistochemistry the expression of CD68+ TAMs and investigated the possible correlation of TAMs with clinical outcome in 64 cases of mature T- and NK-cell lymphomas.

Patients, materials, and methods

Patients and samples

Sixty-four staged and treated patients with mature T- and NK-cell lymphomas between 2000 and 2008 were included in this study in Sun Yat-sen University Cancer Center. Approval for this study was obtained from the institutional review board. Paraffin-embedded tissue blocks from pathology specimens of these cases were collected from the pathological archives of the institution, the source of which included lymph node biopsies and surgical specimens at first diagnosis. All cases had been previously stained with a panel of antibodies, including those to CD3, CD4, CD8, CD5, CD20, CD30, CD45RO, CD56, TIA-1, and granzyme B. Some cases were also stained for anaplastic lymphoma kinase (ALK) and CD10 or in situ hybridization for Epstein–Barr virus-encoded RNA. Each case in the study was reviewed by 2 hematopathologists for confirmation of histological diagnosis according to the World Health Organization criteria for NHL [19]. They were histologically classified as PTCL not otherwise specified (nos) ($n = 28$), extranodal natural killer/T-cell lymphoma (ENKL) ($n = 16$), angioimmunoblastic T-cell lymphoma (AITL) ($n = 10$), and ALK-negative anaplastic large cell lymphoma (ALK-ALCL) ($n = 10$). Baseline clinical information that included sex, age, Eastern Cooperative Oncology Group (ECOG) performance status, Ann Arbor stage, "B" symptoms, lactate dehydrogenase (LDH) levels, extranodal involvement, bone marrow (BM) involvement, bulky disease, and the International Prognostic Index (IPI) was collected at the time of diagnosis. The majority of the patients (92%) were treated with similar therapy protocols based upon combination chemotherapy, typically CHOP or CHOP-like regimens, with radiotherapy if necessary. Data on treatment outcome of chemotherapy were recorded in all 64 cases.

Immunohistochemistry

Immunohistochemical staining with a monoclonal antibody against CD68 (clone KP1, dilution 1:2,000, Dako Cytomation) was applied to 4-mm-thick paraffin-embedded sections in order to identify and quantify TAMs in mature T- and NK-cell lymphomas. In brief, tissue sections were deparaffinized, dehydrated, and then subjected to blocking with hydrogen peroxide, microwave antigen retrieval in 10 mmol/L citrate buffer (pH 6.0), and incubation overnight at 4 °C with the anti-CD68 antibody. The detection was using a standard streptavidin peroxidase technique and chromogen diaminobenzidine. For positive and negative staining controls, we used tissue sections of reactive lymphadenitis known to be immunopositive and tissue sections treated with phosphate-buffered saline instead of primary antibody, respectively.

For enumeration of the number of CD68+ TAMs, five representative fields with the strongest and most uniform staining were examined at low-power magnification (100×). The number of TAMs was then estimated at high-power magnification (400×) from these fields per case. Only CD68+ cells displaying macrophagic morphology were counted. The mean number per high-power field was calculated. Quantification was performed in duplicate by two different pathologists, and then data were averaged and defined as the TAMs content of each case. Cases that varied significantly between the observers were re-evaluated to arrive at a consensus.

Statistical analysis

The mean value of CD68+ TAMs counts was reported. Overall survival (OS) was defined as the time from diagnosis to the date of death due to any cause or to the date of last follow-up evaluation. Mann–Whitney U test or Student's t test was used to assess the correlation between TAMs and clinical parameters. Survival curves were performed using the Kaplan–Meier method, and the log-rank test was used for the comparison of survival differences between groups. The Cox proportional hazard model was used for multivariate analysis. $p < 0.05$ was considered

statistically significant. All statistical analyses were carried out using SPSS software 16.0 (SPSS Inc, Chicago, IL).

Results

Patient characteristics

The clinical and tumor characteristics of all patients are summarized in Table 1. The study group consisted of 20 women and 44 men, with the median age of 51 years (range, 22–78 years). All the patients had performance status (PS) 0 or 1 according to the Eastern Cooperative Oncology Group (ECOG) performance status score. Nearly half of the patients were diagnosed at an advanced stage (stage III or IV). Distribution of the IPI score showed that 36 patients (56.3%) were in the low-risk group (IPI score, 0–1) and 28 (43.7%) were in the high-risk group (IPI score, 2–5). In the entire study group, 59 patients were treated with first-line doxorubicin-containing combination chemotherapy, 38 of whom received CHOP regimen for a median of 4 cycles (range, 2–6). The other five patients were treated with radiotherapy only. With a median follow-up of 43 months (range, 5–102), 39 patients died due to

Table 1 Correlation between TAMs and clinical characteristics of 64 patients with mature T- and NK-cell lymphomas

Clinical characteristics	Cases	Percentage (%)	TAMs content (mean/hpf)	*p*-value
Age				
≤60 years	48	75.0	88.0 ± 48.9	1.000
>60 years	16	25.0	85.3 ± 42.6	
Gender				
Female	20	31.2	70.5 ± 40.6	0.053
Male	44	68.8	95.0 ± 48.3	
Disease staging				
I–II	34	53.1	83.0 ± 48.6	0.350
III–IV	30	46.9	92.3 ± 45.7	
B symptoms				
Present	33	51.6	98.5 ± 57.0	0.046
Absent	31	48.4	75.4 ± 30.2	
Bulky disease				
Yes	13	20.3	76.1 ± 43.6	0.350
No	51	79.7	90.2 ± 48.0	
Bone marrow involvement				
Yes	7	10.9	104.6 ± 61.4	0.384
No	57	89.1	85.2 ± 45.3	
Serum LDH level				
Normal	38	59.4	89.6 ± 47.1	0.769
Above normal	26	40.6	84.0 ± 48.0	
Extranodal involvement				
Yes	44	68.8	90.4 ± 51.4	0.036
No	20	31.2	71.8 ± 31.8	
IPI score				
Low risk (0–1)	36	56.3	83.8 ± 47.3	0.469
High risk (2–5)	28	43.7	91.9 ± 47.3	
Histologic subtype				
PTCL-nos	28	43.8	88.1 ± 41.4	0.396
ENKL	16	25.0	100.2 ± 60.1	
AITL	10	15.6	79.2 ± 36.8	
ALK-ALCL	10	15.6	72.3 ± 49.2	

Abbreviations ALK-ALCL anaplastic lymphoma kinase–negative anaplastic large cell lymphoma, *AITL* angioimmunoblastic T-cell lymphoma, *ENKL* extranodal natural killer/T-cell lymphoma, *IPI* International Prognostic Index, *PTCL-nos* peripheral T-cell lymphoma, not otherwise specified, *LDH* lactate dehydrogenase, *TAMs* tumor-associated macrophages

[a] Student's *t* test, Mann–Whitney *U* test, or Kruskal–Wallis test, as appropriate

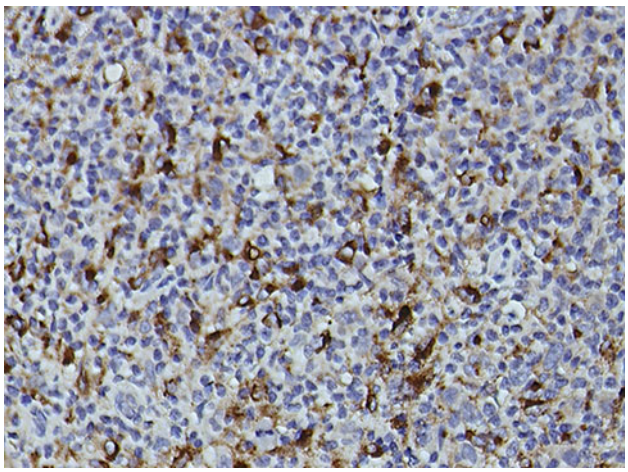

Fig. 1 Representation of high TAMs content in immunostained specimens by the LSAB method (×400)

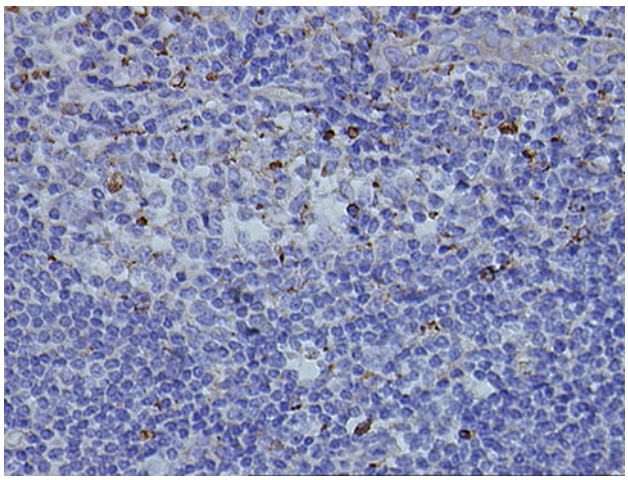

Fig. 2 Representation of low TAMs content in immunostained specimens by the LSAB method (×400)

tumor progression ($n = 36$), treatment complications ($n = 2$), and nonmalignant underlying disease ($n = 1$). The median OS was 30.4 months (95% confidence interval, 8.8–52.0 months). The 1-, 3-, and 5-year OS rates were 68.8, 48.4, and 37.0 %, respectively.

The CD68+ TAMs content and its correlation with clinicopathologic features

The expression of CD68 was detected in the cytoplasm of macrophages in all the 64 cases. Neoplastic lymphoma cells were not stained with anti-CD68 antibody. Most cases of mature T- and NK-cell lymphomas showed CD68+ TAMs infiltrating massively in the stroma (Fig. 1). The mean number of TAMs content of the whole group was 87.2 ± 47.4/high-power field (hpf) (range, 14–225/hpf). Regarding the individual clinicopathologic features in the mature T- and NK-cell lymphomas series (Table 1), higher infiltration of CD68+ TAMs was identified more frequently in patients presenting with B symptoms and with extranodal involvement. No significant difference was observed between TAMs content and age, gender, lymphoma stage, LDH level, IPI risk groups, bulky disease, or bone marrow involvement. The number of infiltrated TAMs in the microenvironment did not differ significantly between different pathological subtypes in our study group.

Survival analysis

On the basis of a previous report [8], we distinguished the patients into two groups according to the levels of CD68+ TAMs content using the mean value of TAMs per hpf as the cutoff point (high TAMs group, >87/hpf; low TAMs group, <87/hpf). The TAMs level was considered low in 36 cases (56.2%) and high in 28 cases (43.8%) (Figs. 1, 2).

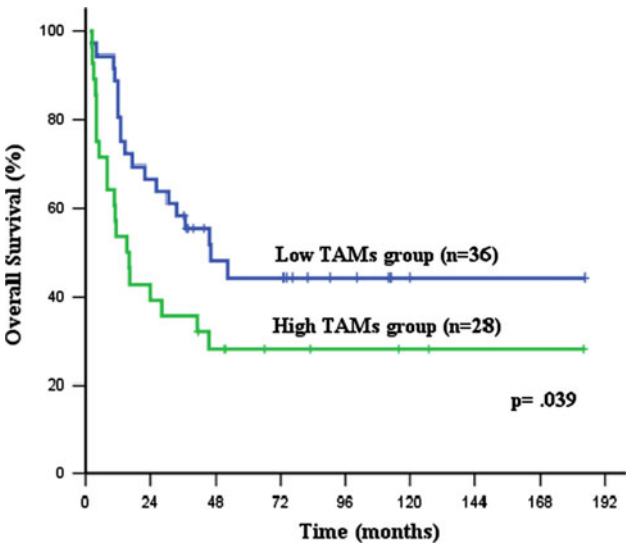

Fig. 3 Survival curves of mature T- and NK-cell lymphomas with respect to the level of TAMs content

The survival analysis and log-rank test showed that patients with high level of TAMs content had a worse 5-year OS compared to those with low level (28.1 vs. 44.3 %, $p = 0.039$) (Fig. 3). Univariate analysis identified high TAMs content, advanced Ann Arbor stages (III/IV), elevated LDH level, IPI score of 2 or higher, B symptoms, and bone marrow involvement as prognostic indicators of poor OS for patients with mature T- and NK-cell lymphomas, as shown in Table 2. No statistically significant differences were identified in OS between different histologic subtypes in our group. A multivariate Cox regression model including TAMs content, IPI score, B symptoms, and bone marrow involvement was applied. High TAMs content was identified as a strong independent predictor of a shorter OS time (RR = 2.464, 95% CI = 1.249–4.861, $p = 0.009$).

Table 2 Prognostic analysis for overall survival of 64 patients with mature T- and NK-cell lymphomas

Variable	*p*-value		HR	95 % CI
	Univariate analysis	Multivariate analysis		
LDH level, above normal versus normal	0.042			
B symptoms, present versus absent	0.047	0.620		
Stage, III–IV versus I–II	0.003			
BM involvement, present versus absent	0.000	0.014	3.751	1.312–10.724
IPI score, high risk versus low risk[a]	0.001	0.037	2.572	1.057–6.259
TAMs content, >87/hpf versus <87/hpf	0.039	0.009	2.464	1.249–4.861

Abbreviations BM bone marrow, *CI* confidence interval, *HR* hazard ratio, *IPI* International Prognostic Index, *LDH* lactate dehydrogenase, *TAMs* tumor-associated macrophages

[a] High risk indicates IPI score of 3–5; low risk indicates IPI score of 0–2

IPI score of 2 or higher and bone marrow involvement were also shown to be independent prognostic factors for OS ($p = 0.037$, $p = 0.014$, respectively) (Table 2).

For the 38 patients who received frontline CHOP chemotherapy, the prognostic effect of TAMs content on the outcome was investigated. In this subgroup, patients with high level of TAMs had significantly shorter OS than those with low level (5-year OS, 26.3 vs. 54.4 %; $p = 0.027$) (Fig. 4). We also analyzed the prognostic significance of TAMs expression in the same histological subtype. Among the 28 PTCL-nos cases studied, the 5-year OS of patients with high infiltration of TAMs was 30.8% compared with 44.4 % in patients with low infiltration ($p = 0.095$). Patients with ENKL in our study did not exhibit significant differences in 5-year OS based on the level of CD68+ TAMs content (high TAMs group, 33.3%; low TAMs groups, 30%; $p = 0.85$).

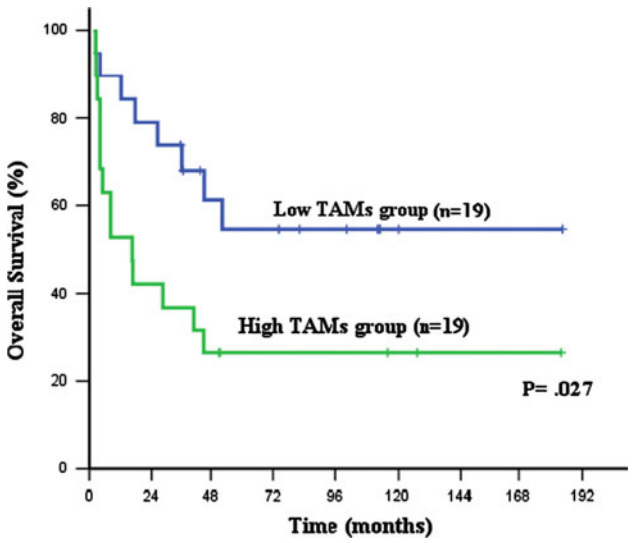

Fig. 4 Survival curves of CHOP-treated patients with mature T- and NK-cell lymphomas according to the level of TAMs content

Discussion

As a major component of the immune infiltrate seen in a variety of lymphoma subtypes, TAMs have phenotype more similar to the M2-type macrophages and exhibit protumoral functions, including poor cytotoxicity for tumor cells, expression of growth factors, promotion of tumor angiogenesis, and suppression of adaptive immunity [3–5, 10]. The role of TAMs in determining clinical behavior and prognosis has been widely investigated in FL and DLBCL [7–14]. However, the role of macrophages in T-cell lymphoma is largely unexplored. And only a few studies focused on the prognostic value of TAMs in the stromal microenvironment of mature T- and NK-cell lymphomas. Wilcox et al. [20] provided preclinical evidence that monocytes, which are the origin of TAMs [4], promoted the growth and survival of malignant T cells directly and indirectly, by preventing dendritic cells maturation. Zhang et al. [21] elucidated the expression of CD68+ TAMs in 38 cases of PTCL-nos by immunohistochemistry, concluding that high level of TAMs content was a predictor of poor outcome in patients with PTCL-nos. Another immunohistochemical study of 42 patients with AITL found no correlation between the number of CD68+ cells and OS [22].

In the current study, we investigated the expression of CD68+ TAMs in the four common aggressive subtypes of mature T- and NK-cell lymphomas. No significant difference in TAMs content was observed among histologic subtypes, although ENKL showed the highest mean number of infiltrated macrophages. Higher infiltration of TAMs was found related to unfavorable baseline clinical factors such as B symptoms and extranodal involvement, indicating that TAMs might promote the development and progression of lymphoma. On the basis of the TAMs content, patients were divided into high TAMs group and low TAMs group in this series. Survival analysis revealed that high TAMs group had worse 5-year OS than low TAMs group. In histological subgroup analysis, a trend toward a

longer survival for patients with low TAMs content was observed only in the PTCL-nos subgroup, although without statistical significance, which might be due to the limited sample size of this subtype (28 cases). Most patients in our study were primarily treated with anthracycline-based chemotherapy. Considering that specific therapeutical approaches may influence the prognostic impact of TAMs, patients uniformly treated with first-line CHOP chemotherapy were also analyzed. And the result was similar to that of the whole group. Despite a small sample size, the present study demonstrated an inverse correlation between the infiltration of stromal TAMs and survival duration of CHOP-treated patients with mature T- and NK-cell lymphomas.

In multivariate analysis in the whole group, TAMs content remained an independent biological variable for survival distinct from the IPI that describes merely clinical indicators. The combination of TAMs with the clinical prognostic model IPI may better stratify patients with mature T- and NK-cell lymphomas for novel and risk-adapted therapeutic options. These results not only show consistent with the report of Zhang et al. [21], but also agree with previous observations in FL and DLBCL, suggesting that macrophages, as one of the key elements in host immune response, may affect the clinical course and survival of patients [7–14]. Considering the immunological nature of lymphoma, the interplay between the immune-related macrophages and malignant lymphoid cells may play an important role in the pathogenesis and clinical aggressiveness of both B-cell and T-cell NHL.

Results of our study should be interpreted within its limitations, such as retrospective retrieval of data and inclusion of patients with different histological subtypes of mature T- and NK-cell lymphomas. Further prospective research should be conducted on each specific subtype with larger sample sizes so as to validate the prognostic relevance of TAMs in mature T- and NK-cell lymphomas. As to the immunohistochemical marker for TAMs, some reports considered that CD163 expression was more specific to the activated phenotype of macrophages compared with CD68 [14, 22, 23]. The number of CD163+ macrophages was found inversely correlated with OS to some extent in AITL [22]. Nevertheless, it is still under debate which marker should be chosen to reliably identify TAMs and which one provides better information for outcome prediction.

In summary, our study demonstrated the prognostic value of enumerating TAMs in the microenvironment of mature T- and NK-cell lymphomas. It showed that high level of TAMs content indicated an adverse overall outcome. It is reasonable to infer that the stromal macrophages might be involved in the genesis and progression of T-NHL. Further studies including larger groups of patients with specific histologic subtypes are warranted to confirm our findings. Additional experimental research focusing on the phenotypes and functions of macrophages will also be necessary in order to corroborate the biological basis of these findings.

Acknowledgments We thank all the treating physicians in the Department of Medical Oncology, Cancer Center of Sun Yat-Sen University, for their assistance.

Conflict of interest The authors declare no conflict of interest.

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