Tumor-associated macrophages in diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of lymphoma and affected patients' outcomes vary from the extremes of cure in the majority of patients to the dismal prognosis of primary refractory and, to a lesser extent, relapsed disease that occur in 30-40% of cases. The understanding of tumor and host factors that contribute to treatment failure is, therefore, a critical hurdle that needs to be overcome in order to therapeutically target the mechanisms underlying treatment resistance and eventually improve survival.

DLBCL is characterized histologically by sheets of large, transformed B cells that efface the normal lymph node architecture.[1] However, tumor samples contain varying proportions of admixed benign cells that include various subsets of T cells, macrophages, mast cells and stromal cells and collectively define the microenvironment.[2] Evidence exists in the literature to substantiate the concept that, in most instances, DLBCL is not purely the result of autonomous cell growth but also relies on survival and proliferation signals from the microenvironment.[2] The relevance of the microenvironment for tumor control in this disease is exemplified by the selection of recurrent genetic alterations that mediate escape from immune surveillance.[3,4] With regards to patients' outcomes, the contribution of the tumor microenvironment to treatment failure in DLBCL was highlighted for example by a study from the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) in which two gene expression signatures derived from the microenvironment - the "stromal-1" and "stromal-2" signatures - were predictive of favorable or unfavorable survival, respectively, in patients treated with CHOP alone or CHOP in combination with rituximab.[5] The "stromal-1" signature is thought to arise from extracellular-matrix deposition and histiocytic infiltration whereas the "stromal-2" signature reflects tumor blood-vessel density.[5] The various cellular components of the microenvironment have also been studied using immunophenotypic techniques such as immunohistochemistry or flow cytometry, but it is fair to state that thus far, no microenvironment-derived biomarker has proven to predict patients' outcomes with sufficient reproducibility to warrant inclusion into the routine diagnostic work-up.

In this issue of the journal, Riihijärvi *et al*. describe the association of CD68 mRNA levels and CD68 protein expression with outcome in DLBCL.[6] The cohorts under study include patients with gene expression data from a Nordic phase II trial (dose-dense chemoimmunotherapy with central nervous system prophylaxis, n=38),[7] from the Cancer Genome Characterization Initiative (R-CHOP, n=92) and from the LLMPP (R-CHOP, n=233 and CHOP, n=181). Immunohistochemistry data were generated using the KP1 anti-human CD68 antibody and positive cells were counted manually. The immunohistochemistry cohorts consisted of cases from the above-mentioned Nordic phase II trial (n=59) and population-based DLBCL cases (chemoimmunotherapy, n=72 and non-rituximab-based therapy, n=50). Although some of the cohorts were small, the thresholds for discriminating high from low expressers were variable and associations with outcome were not universally statistically significant, the findings are remarkably consistent throughout the study by Riihijärvi *et al.*: CD68 was associated with a favorable prognosis when patients were treated with rituximab in addition to multi-agent chemotherapy, and with a poor outcome when rituximab was not given. The prognostic implication of tumor-

Table 1. Summary of published studies assessing the prognostic implication of tumor-associated macrophages in DLBCL.

Marker	Technique	Number of patients	Treatment	Outcome correlation	Reference
CD68	IHC	176	Variable, no rituximab	Not significant (PFS, OS)	Hasselblom *et al.*, 2008[16]
SPARC CD68	IHC	262	R-CHOP R-CHOP-like	SPARC favorable (EFS, OS) CD68 not significant (EFS, OS)	Meyer *et al.*, 2011[17]
CD68	IHC	112	CHOP	Adverse (PFS, OS)	Cai *et al.*, 2012[15]
CD68/HLA-DR CD68/CD163	Double IHC	101	R-chemo	M1 (CD68/HLA-DR): not significant (OS) M2 (CD68/CD163): adverse (OS)	Wada *et al.*, 2012[19]
CD68	IHC	309	R-CHOP (161)	R-CHOP: not significant (PFS, OS)	Coutinho *et al.*, 2014[18]
CD68/HLA-DR CD68/CD163	Double IF	61	R-CHOP (57)	M1 (CD68/HLA-DR): not significant (DFS, OS) M2 (CD68/CD163): adverse (DFS, OS)	Marchesi *et al.*, 2014[20]
CD68	IHC	29	R-CHOP	Adverse (non-response)	Marinaccio *et al.*, 2014[24]
CD68 CD163	IHC	165	R-CHOP (109)	CD68, CHOP: adverse (OS) CD68, R-CHOP: favorable (OS) CD163, R-CHOP: adverse (PFS, OS)	Nam *et al.*, 2014[8]
CD68	GEP IHC	38+92+233+181 (GEP) 59+72+50 (IHC)	R-chemo Chemo	R-chemo: favorable (PFS, OS) Chemo: adverse (PFS, OS)	Riihijärvi *et al.*, 2014[6]
CD68 CD163	IHC	36	R-CHOP	CD163: not significant (PFS)	Yamamoto *et al.*, 2014[25]

DLBCL: diffuse large B-cell lymphoma, IHC: immunohistochemistry, PFS: progression-free survival, OS: overall survival, EFS: event-free survival, IF: immunofluorescence, DFS: disease-free survival, GEP: gene expression profiling.

associated macrophages does, therefore, appear to be dependent on the administration of anti-CD20-directed antibodies. Using immunohistochemistry, Riihijärvi *et al.* also studied the expression of CD163, a marker for alternatively polarized/tumor-promoting macrophages, and various other microenvironment-related markers, but did not find any further associations with outcome, despite significant correlations between CD68 and CD163, both at the mRNA and protein levels. Lastly, it is relevant to note that CD68 expression was not associated with germinal center or non-germinal center molecular subtypes, as identified by the Hans algorithm.

The modulation of the prognostic effect of CD68-positive macrophages by rituximab, as reported by Riihijärvi *et al.*, is in line with the work by Nam *et al.*[8] and mirrors similar observations in follicular lymphoma.[9,10] Taken together, these studies raise the question of the mechanism that underlies the reported outcome correlations. Rituximab exerts its therapeutic role through three main modes of action after binding to CD20 on B cells: complement activation, antibody-dependent cellular cytotoxicity/phagocytosis and the induction of intra-cellular signaling resulting in cell death.[11] The exact contribution of each of these mechanisms *in vivo* is a matter of debate, but it has been shown that Fcγ receptors, which bind the constant fragment of antibodies, are required for rituximab-mediated lymphoma killing.[12] These receptors are expressed on a variety of cells that include macrophages, clodronate-mediated depletion of which nullifies the therapeutic activity of rituximab in mice.[12] Likewise, Fcγ receptor polymorphisms that modulate the affinity of binding to IgG subclasses have been reported to affect the efficacy of rituximab used as a single agent.[13] Binding of anti-CD20 antibody-coated tumor cells to Fcγ receptors on effector cells does, therefore, appear to be important and a higher number of cells harboring Fcγ receptors might increase the likelihood of such an interaction taking place. Taken to its extreme, this line of thought would provide the rationale for interventions that increase the number of tumor-associated macrophages in those patients whose tumors are infiltrated by rare macrophages only. To this effect, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been studied in addition to rituximab alone or in combination with chemotherapy in several small, non-controlled trials, both in indolent and aggressive lymphomas.[14] Although promising responses have been reported, it is not possible to draw definite conclusions regarding the efficacy of such an approach without larger, randomized and controlled trials. In addition, GM-CSF has pleiotropic effects,[14] and any clinical benefit can be explained by mechanisms other than an increase in macrophage function or number.

The study by Riihijärvi *et al.* builds on the existing literature and adds texture to a controversial field (Table 1). Whereas Nam *et al.*[8] reported similar findings, as mentioned above, and the study by Cai *et al.*[15] also showed that CD68 is a marker of poor outcome in CHOP-treated DLBCL patients, the studies by Hasselblom *et al.*,[16] Meyer *et al.*[17] and Coutinho *et al.*[18] did not reveal significant associations between CD68 protein expression and survival. Furthermore, Wada *et al.*[19] and Marchesi *et al.*[20] reported that an M2 macrophage phenotype, as defined by double staining for CD68 and CD163, is associated with adverse outcome in R-CHOP-treated patients, whereas an M1 phenotype is not. How can these discrepancies be explained? First of all, it is noteworthy that the cited studies vary in their design by factors such as cohort size, antibody clone, scoring method (manual *versus* automated, tissue microarray *versus* individual sections) and treatment received. Inter-laboratory differences in staining techniques and protocols contribute, generally speaking, to the poor reproducibility and difficulties in standardizing immunohistochemistry-based biomarkers that can easily explain shifts in signal distribution between cohorts, introducing noise in the assessment of whether a given case falls below or above a predefined threshold. Moremore, certain anti-CD68 antibodies, notably clone KP1, stain other cellular elements such as myeloid and endothelial cells.[21] Lastly, it has been suggested that host lifestyle factors such as smoking and low body mass index are correlated with higher levels of CD68 staining, at least in follicular lymphoma.[22] These considerations could play a role in explaining discrepant results between different cohorts of patients. For all those reasons, CD68 cannot be recommended for immediate implementation in the clinic as a prognostic biomarker in DLBCL.

Beyond the prognostic implication of CD68 expression, several other key questions have not been completely addressed in the literature to date. The main unknowns are the mechanism by which macrophages are recruited into the DLBCL microenvironment and the biology underlying the inter-patient variability in the extent of macrophage infiltration. In follicular lymphoma, stromal cells derived from mononucleated cells have been shown *in vitro* to secrete CCL2, thereby recruiting monocytes and differentiating them into a tumor-promoting phenotype.[23] A similar insight into macrophage biology is currently lacking in DLBCL. Another question of interest is whether the observed outcome correlations will hold true for other anti-CD20 antibodies and whether the principle of increased antibody-dependent cellular cytotoxicity/phagocytosis of opsonized tumor cells in the presence of macrophages can be generalized to all malignancies that are amenable to monoclonal antibody-based therapy. Future studies addressing these key questions are needed to expand on the observations of Riihijärvi *et al.* and fully understand the impact of rituximab-containing treatment regimens on tumor microenvironment interactions in DLBCL.

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Inherited thrombocytopenias in the era of personalized medicine

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Until 15 years ago, inherited thrombocytopenias (ITs) were quite an indistinct group of disorders, only a few forms of which had been clearly defined. Moreover, the genetic defect was known for only two disorders: Bernard-Soulier syndrome (BSS) and Wiskott Aldrich syndrome (WAS).

Since then, our knowledge of ITs has greatly advanced and we currently know at least 21 genes whose mutations result in 19 disorders (Table 1). The study of large series of patients identified the particular characteristics of the different forms and revealed that they have different degrees of clinical complexity and a great variation in prognosis. Furthermore, we realized that different mutations in the same gene may cause from different phenotypes. Finally, specific treatments for specific disorders have been identified, and given this, we are now truly in an era in which personalized medicine can play a role in the treatment of ITs.

Molecular characterization for defining prognosis

For a long time, the most frequently diagnosed form of IT was BSS, which typically presents from birth with recurrent hemorrhage.[1] Bleeding tendency is usually severe also in the other well-known ITs, such as WAS, congenital amegakaryocytic thrombocytopenia (CAMT), and gray platelet syndrome (GPS). A major concern in these patients has always been to prevent bleedings from hemostatic challenge and to stop spontaneous hemorrhage.

In the last few years, our understanding of ITs has