ommendations are that if a matched unrelated donor can be found quickly, then transplantation could be considered a potential first-line option in those children who lack a matched sibling donor. The decision to proceed with horse ATG-based IST or an upfront transplant from a matched unrelated donor will, therefore, depend on the preferences of patients and physicians and donor availability until further data become available.

Conclusion

Survival outcomes for low-risk RCC and acquired SAA in children following IST with horse ATG/cyclosporine are excellent. Future strategies will now need to focus more on quality of life and failure-free survival so that further improvements can be made. This can only be achieved with well-designed prospective clinical studies.

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Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

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Assessing the prognostic impact of immune cell infiltrates in follicular lymphoma

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Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma (NHL) subtype in the Western world. Even without specific therapy, many patients show prolonged survival, but over time a significant proportion of patients progress to aggressive, often therapy-refractory and ultimately fatal disease. Although clinical tools such as the FL International Prognostic Index,[1] based on simple clinical parameters, allow risk stratification, the clinical course of FL in an individual patient is unpredictable, and the search for better tissue-based prog-

nosticators is ongoing.[2] In addition to intrinsic properties of the tumor cells, such as genetic alterations and epigenetic modifications, host factors including the lymphoma microenvironment play a significant role in disease evolution.

The role of the microenvironment in follicular lymphoma

The importance of stromal cells and reactive immune cells in FL may already seem evident from its unique growth pattern and cell composition. FL recapitulates many features of the normal B-cell follicle with follicular dendritic cell networks, T cells, including the follicular T-helper subset, and macrophages, which together make up a significant proportion of the cellular infiltrate, sometimes surpassing the number of neoplastic cells. In accordance with their microenvironment, FL cells are permanently locked in a germinal center stage of differentiation with expression of germinal center markers such as CD10, BCL-6, LMO2 and GCET, and ongoing somatic hypermutation of the rearranged immunoglobulin genes.[3,4] Despite the constitutional expression of the BCL-2 oncoprotein preventing apoptosis, FL cells nevertheless need, at least initially, signaling from their microenvironment for proliferation and expansion, as well as protection against an attack from the immune system. The mechanisms of this supportive signaling are only partly understood. B-cell receptor engagement by low affinity antigen binding, as well as lectin-mediated interaction with surrounding reactive cells based on sugar residues attached to novel N-glycosylation sites, which are created by somatic hypermutation of IG molecules, might play a role.[5,6] Follicular T-helper (FTH) cells, regulatory T cells (Treg), follicular dendritic cells and macrophage subsets, as well as their secreted cytokines, provide a nurturing environment and protection. In contrast, other subpopulations such as cytotoxic T cells may be able to keep the tumor in check for a prolonged time.[4,7] The critical role of the cellular composition of the microenvironment *versus* the intrinsic properties of tumor cells for FL prognosis was first demonstrated in a landmark study by Dave *et al.*, which identified two different gene expression signatures derived from stromal cells by expression profiling, designated immune response-1 (IR-1) and immune response-2 (IR-2),

Table 1. Selected immunohistochemical studies on the prognostic impact of the microenvironment in follicular lymphoma.

Authors/Ref.	Patient number	Therapy	Tissue/ quantification method	Main prognostic impact	Comments
Alvaro *et al.*[11]	211	Chemo (various)	TMA/ pattern assessment and image analysis	Low CD8+ poor	Only high FLIPI associated with poor survival in multivariate analysis
Alvaro *et al.*[10]	211	Chemo (various)	TMA/ pattern assessment and image analysis	STAT1+ macrophages poor	Same cohort as Alvaro *et al.*,[11] only CD68 and STAT1 examined
Canioni *et al.*[12]	194	Chemo+/-Rituximab	WS/counting; intra- and extrafollicular	Low CD68+ good (Chemo only)	Prognostic effect (EFS) abolished in rituximab arm
Carreras *et al.*[13]	97	Heterogeneous (CT+/-Rituximab, RT)	WS/ image analysis	High FOXP3+ good	Low FOXP3 counts associated with transformation
Carreras *et al.*[14]	100	Heterogeneous (CT+/-Rituximab, RT)	WS/ image analysis	High PD1+ good	Low PD1 counts associated with transformation Same cohort as Carreras *et al.*[13]
De Jong *et al.*[15]	61	Fludarabine *vs*. CVP	TMA/ pattern assessment and semiquantitative	High FOXP3+perifollicular good	CD68 and FDC meshwork with divergent results according to treatment arm
Farinha *et al.*[17]	99	Chemo + RT	TMA/ pattern assessment and counting	High CD68+ macrophages poor	
Farinha *et al.*[16]	105	Chemo	TMA/ pattern assessment and counting	FOXP3+ follicular poor FOXP3+ diffuse good	Quantity of T-cell subtypes NS for survival
Glas *et al.*[9]	58	Chemo/RT	WS/ pattern assessment and semiquantitative	CD4+ intrafollicular poor CD4+ perifollicular good	Patient selection transformed/not transformed
Laurent *et al.*[19]	80	R-chemo	WS/ pattern assessment and semiquantitative	High Granzyme B+ good	Intermediate/high FLIPI
Lee *et al.*[20]	59	Heterogeneous (Chemo, RT)	TMA/ pattern assessment and semiquantitative	High CD4+ good FOXP3+ perifollicular good	Patient selection according to long/short survival
Sweetenham *et al.*[21]	180	CT+/-Rituximab, Radioimmunotherapy	TMA/ pattern assessment and counting	None (FOXP3+ and CD68+)	Patients from 3 clinical trials
Taskinen *et al.*[22]	141	Chemo+/-Rituximab	WS and TMA/counting	High CD68+ poor (CT only) High CD68+ good (CT+Rituximab)	Two different cohorts +/- Rituximab CD3 NS for survival
Wahlin *et al.*[23]	70	Heterogeneous	TMA/ image analysis	High CD4+ poor High CD8+ good High PD1+ good FOXP3+ follicular good CD68 perifollicular poor	Patients selected according to extreme outcomes

WS: whole section; TMA: tissue microarray; CVP: cyclophosphamide, vincristine, prednisone; RT: radiotherapy; NS: not significant.

respectively.[8] IR-1 was enriched for T-cell-derived genes and some genes strongly expressed in monocytes and macrophages and correlated with improved survival, whereas IR-2 was enriched for genes expressed in macrophages and dendritic cells and predicted poor survival. Although other GEP studies in part gave conflicting results,[9] the prognostic impact of infiltrating immune cells in FL has remained a focus of research. Many subsequent efforts were directed at translating gene expression-defined signatures derived from the reactive microenvironment into more practical and cost-effective tools, using immunohistochemical identification and quantitation of immune cells in tissue sections of paraffin-embedded FL samples as surrogate marker.

Microenvironment composition and prognosis

In the last decade, a significant number of studies have addressed the prognostic impact of macrophage content, as well as density and distribution of T cells and T-cell subsets, including $CD8^+$ cytotoxic cells, $CD4^+$/$FoxP3^+$ Treg and $CD57^+$ or PDCD-1(CD279)$^+$ TFH cells. Furthermore, other cell types, such as mast cells or microvessels, were correlated with survival.[9-23] Disappointingly, and as summarized previously by de Jong *et al.*,[15] no clear picture has emerged from these studies so far.[2]

The reasons for the failure of these studies to give consistent results in terms of prognosis are manifold, including heterogeneity in study design and end points, patient selection and technical aspects of immune cell quantification (Table 1). As shown previously, different chemotherapy protocols and addition of the anti-CD20 antibody rituximab profoundly impact the prognostic relevance of the reactive cell infiltrate in FL.[12,15,22] Furthermore, the antibody panels used in these studies do not reflect the functional complexity of immune cell subsets and are unable to capture, for example, the polarization of macrophages into M1 and M2 subtypes with contrasting effect on FL cell proliferation, or differences in TFH cell subsets.[4]

However, the contradictions concerning the prognostic impact of microenvironment composition may also be due to more prosaic technical factors, such as representativity and fixation quality of tissue samples, lack of standardization of immunostaining protocols and antibodies, use of whole tissue sections *versus* tissue microarrays and different quantification protocols. Furthermore, a much cited but poorly investigated factor of potentially critical impact is the inter- and intraobserver variability in the semi-quantitative estimation or quantitative assessment of immunostained cells in tissue sections under the microscope. Another aspect that is handled in different ways in the studies cited above is microtopography, with only some of the studies taking the intra- *versus* perifollicular localization of evaluated reactive cell types into account.

In this issue of *Haematologica*, the Lunenburg Lymphoma Biomarker Consortium, an assembly of nine international lymphoma collaborative groups, has undertaken to investigate the variability of visual estimates of immune cell infiltrates in FL by pathologists, since this may significantly affect the outcome of studies assessing the prognostic impact of the microenvironment.[24] Using a tissue array of newly diagnosed FL cases from a single institution with available flow cytometry (FCM) results, slides were stained in a central laboratory for several T-cell markers, antigens expressed on the lymphoma cells, and markers for other components of the microenvironment including macrophages and microvessel density. The same immunostained sections were circulated among the participating laboratories and evaluated according to pre-defined criteria. Results obtained by visual estimation were compared among the different laboratories, as well as to the results of computerized image analysis performed in two of the institutions with the same hardware, but distinct algorithms, and to the data obtained by FCM. Not really surprisingly, the study demonstrated a high variability among estimates between different participating institutions with only moderate to poor concordance between the laboratories for most markers, and with the results of image analysis and FCM. Even though some laboratories did better than others in terms of comparability with the other techniques, the levels of concordance achieved by the group as a whole demonstrate that these results are not good enough to be used as robust prognostic marker. This is an important message for similar biomarker studies to come.

A drawback of the study, however, is the lack of inclusion of a manual counting arm. "Eyeballing", that is the visual estimation of percentages of immunostained cells in tissue sections, even if performed after training and according to pre-set rules as done in this study, is likely to be significantly influenced by hard-to-control variability in estimation, e.g. depending on the intensity of the immunostains or distribution patterns. Manual counting, in contrast, if performed under controlled conditions, can provide fairly reliable results also in a multicenter setting and in comparison to image analysis.[25] It is understandable that a manual counting arm would have increased the amount of work for the study participants significantly, but this widely used and generally accessible technique for daily routine diagnostics should still not be dismissed altogether.

Another important contribution of this paper is the direct comparison of FCM and section-based assessment of immune cell infiltrates, using both visual estimation and image analysis. Surprisingly, relatively few previous studies have addressed this issue.[23] For lymphomas, FCM is a well-established technique not only to determine the phenotype of the neoplastic population, but also to assess the percentage of reactive cells. Interestingly, the authors of the present study have documented a constantly higher proportion of reactive cells by image analysis in comparison to FCM. Nevertheless, this overestimation remained constant, in contrast to the more random distribution of the visual estimates. Since the absolute counts are of less importance than the inter-individual differences, this discrepancy is probably irrelevant for the determination of the prognostic impact of immune cell infiltrates.

Is FCM the answer for assessing the cellular composition in a more objective way? Possibly yes, but in contrast to the practice in the USA, where most lymph node samples are submitted to FCM in addition to histological examination, this is not the case in many European countries. In addition, the representativity of the specimen submitted to FCM remains an issue. Therefore, image analysis of immunostained sections is likely the easier way to assess the cellular composition of the microenvironment in FL, since it also allows unrestricted evaluation of archival specimens.

Future perspectives

Another important aspect of FL biology conspicuously underrepresented in the literature is the interaction between genetic and other molecular features of the tumor cells, composition of the microenvironment and prognosis. Is a tumor-suppressive microenvironment able to keep FL cells with high-risk molecular features in check for a prolonged time? To what extent do FL cells govern the expression profile and functional properties of an accompanying reactive cell?[18] Does the molecular profile of FL cells have an impact on the composition of the reactive cellular infiltrate, or is this exclusively determined by the state of the host's immune system? These and other questions need to be answered in order to fully understand the role of the microenvironment in the biology of FL.

In summary, the work by the Lunenburg Lymphoma Biomarker Consortium clearly documents the way hematopathology has to go forward in order to bring tissue-based quantitative biomarkers into clinical practice: standardization has to accompany every step, from selection of the type of material (whole section *vs.* tissue microarrays), antibodies and immunostaining protocols to quantification. It is evident from this and a considerable number of previously published studies that estimation by "eyeballing" is not good enough, especially if it comes to comparison between different centers or studies, or if the results are used as a part of the clinical decision-making process. Image analysis is certainly an important step on the way to reliable immunohistochemical biomarkers, but needs to be complemented by equally stringent efforts directed at standardizing the other aspects of tissue section-based biomarker studies.

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Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

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