PROGNOSTIC INFORMATION FROM NONMALIGNANT AND MALIGNANT LYMPHOCYTES IN FOLLICULAR LYMPHOMA IN RELATION TO THERAPY

Björn Engelbrekt Wahlin
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And whatever your labors and aspirations,
in the noisy confusion of life, keep peace in your soul.
With all its sham, drudgery, and broken dreams,
it is still a beautiful world.
Be careful. Strive to be happy.

Max Ehrmann, the last stanzas of Desiderata

For Ylva, Astrid and Harald
ABSTRACT

Follicular lymphoma is the most common indolent lymphoma. It is composed of centrocytes and centroblasts, residing in follicles that also harbour nonmalignant immune and stroma cells. Follicular lymphoma is graded according to the World Health Organization criteria that are based on the frequency of centroblasts. There is consensus that grades 1 and 2 are indolent, but not whether grade 3 is aggressive. Differences between grades 3A and 3B are also unclear. The nonmalignant cells in the microenvironment interact with the tumour cells and with each other. These interactions may be important for disease outcome. Since the introduction of the therapeutic monoclonal anti-CD20 antibody rituximab, the prognosis of follicular lymphoma has improved. It is likely that the mechanisms of rituximab affect and involve not only CD20+ follicular lymphoma cells but also the surrounding as well as the systemic immune cells. The aim of this thesis was to identify biological predictors for outcome in follicular lymphoma in relation to therapy.

In paper I, using flow cytometry, we reported that higher numbers of CD8+ T cells in diagnostic lymph nodes are an independent predictor of better overall and disease-specific survival. This finding was reproduced in paper II in which computerised quantifications of tissue microarrays were used for a unifying multivariate model. This model showed that many cells in the microenvironment were independently important for outcome. Higher levels of cells positive for CD8 (cytotoxic T cells), forkhead box protein 3 (regulatory T cells) and programmed death-1 (PD-1+ T cells) correlated with good prognosis, but higher levels of cells positive for CD4 (helper T cells) and CD68 (macrophages) with poor. The best predictors for poor outcome were increasing CD4/CD8 and follicular/interfollicular CD4 ratios, suggesting that outcome is influenced by the balance between detrimental follicular B-helper and helper2 T-cells on one hand and favourable cytotoxic and helper1 T cells on the other. In paper III we used prospectively recorded flow cytometry analyses from two randomised trials where all patients received single rituximab with or without interferon-α priming. T cells in tumours (both CD4+ and CD8+) were associated with fast and good clinical responses to rituximab, while T cells in blood (both CD4+ and CD8+) correlated with slower but good and sustained responses, and were more important for survival. Interferon-α abrogated the dependence on high numbers of CD8+ cells (in both blood and tumours) for good rituximab responses. In paper IV we reviewed the follicular lymphoma grades in 828 patients with long follow-up times, of whom 40% received upfront rituximab. Compared with grade 1–3A patients and independently of clinical factors, grade 3B patients showed higher mortality but outcome was improved after upfront anthracyclines. Grade 3B patients experienced no relapses or deaths beyond five years of follow-up. Furthermore, patients with grade 3B were different in their clinical characteristics. In the entire population, patients with grade 3A had similar outcome as those with grade 1–2. However, in patients given upfront rituximab-containing therapy, increasing grades of 1, 2, and 3A correlated with better overall survival and time to treatment-failure, independently of clinical factors.

We conclude that outcome in follicular lymphoma is determined by the balance between competing immune cells in the microenvironment and by their interactions with each other and with the tumour cells. Rituximab and interferon-α alter the prognostic properties of the immune cells, and also involve systemic T cells that may be very important for disease outcome. Grade 3B, or follicular large B-cell lymphoma, is a distinct, aggressive but curable entity. Grades 1, 2 and 3A are indolent and incurable. Increasing grades predict better outcome with rituximab therapy. Our findings suggest a future of personalised therapy based on biological characteristics of the patients and of the tumours.
LIST OF PUBLICATIONS


# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>BCL</td>
<td>B-cell CLL/lymphoma</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell (immunoglobulin) receptor</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40-ligand</td>
</tr>
<tr>
<td>CHOP</td>
<td>Cyclophosphamide-doxorubicin-vincristine-prednisone</td>
</tr>
<tr>
<td>CR, PR, MR</td>
<td>Complete, partial and minor response</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>CVP</td>
<td>Cyclophosphamide-vincristine-prednisone</td>
</tr>
<tr>
<td>CXCR5</td>
<td>C-X-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CXCL13</td>
<td>C-X-C motif chemokine 13 (B lymphocyte chemoattractant)</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>EV-1, EV-2</td>
<td>First and second treatment response evaluation</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FLIPI</td>
<td>Follicular lymphoma international prognostic index</td>
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<tr>
<td>FOXP3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>hpf</td>
<td>High-power field (0.159 mm²)</td>
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<tr>
<td>ICOS</td>
<td>Inducible costimulator</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGH</td>
<td>Heavy immunoglobulin chain</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>NLG</td>
<td>Nordic Lymphoma Group</td>
</tr>
<tr>
<td>PD, SD</td>
<td>Progressive and stable disease</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>R-</td>
<td>Rituximab combined with</td>
</tr>
<tr>
<td>SAP</td>
<td>Signalling lymphocyte activation molecule-associated protein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>Follicular B-helper T-cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;H1&lt;/sub&gt;, T&lt;sub&gt;H2&lt;/sub&gt;</td>
<td>Helper1 and helper2 T-cells</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
1 AN INTRODUCTION TO FOLLICULAR LYMPHOMA

1.1 HISTORY

1.1.1 Brill–Symmers’ disease

In 1925 Brill, Baehr and Rosenthal reported the cases of two patients with a hitherto undescribed disease of enlarged lymph nodes with macroscopically visible follicles, “some as large as a pinhead.” Microscopically, the picture was equally striking, not easily confused with ordinary follicular hypertrophy. “Individual follicles may occupy several microscopic fields. [...] They are seen to lie in such close contact with one another that little or no intervening pulp is visible in most parts of the node, and the lymph sinuses are for the most part obliterated...” (Figure 1-1).

![Figure 1-1: Follicular lymphoma. Brill, Baehr and Rosenthal’s picture of a lymph node under low magnifying power, showing "gigantic enlargement of the lymph follicles" is the first of a follicular lymphoma. (Reproduced with permission from the American Medical Association.]

The disease was separately described by Symmers in 1927. Hence it was named Brill–Symmers’ disease. The disease was reported as being indolent and, as later authors noted, “Symmers at first did not believe that the condition was neoplastic at all and later conceded this point with considerable reservation.”
1.1.2 Capriciousness and a lymphoma revealed

The malignant nature of Brill-Symmers’ disease was recognised from its propensity to transform to aggressive lymphosarcoma, which corresponds to diffuse large B-cell lymphoma (DLBCL) in present-day terminology. Instead of the eponym, Gall and Mallory promoted the term follicular lymphoma in their 1942 publication that became used as a diagnostic classification guide for lymphomas. In those days the average period of survival after diagnosis was about five years but with large individual variation.

1.1.3 The first therapeutic approaches

In the 1940s follicular lymphoma was treated with radiation in large fields, usually low-dose but higher doses were given in transformed cases. In the few patients with localised disease, cure was attempted with surgical excision of the engaged lymph nodes.

1.1.4 Discovering the origin of follicular lymphoma: B cells with t(14;18)

In 1974 follicular lymphoma was recognised as a tumour originating from germinal centre B-cells. Nine years later the hallmark chromosomal aberration of follicular lymphoma, the t(14;18)(q32;q21) was identified. The B-cell CLL/lymphoma (BCL)-2 gene at 18q21 is juxtaposed to the heavy immunoglobulin chain (IGH) gene at 14q32, placing it under the control of the IGH-E\(\mu\) enhancer, leading to its deregulated expression and to constitutively high levels of the BCL-2 protein, which prevents programmed cell death (apoptosis). The t(14;18), seen in up to 90% of all follicular lymphomas, is necessary but not in itself sufficient for tumorigenesis. Follicular lymphomas negative for t(14;18) differ in gene expression profiles but not in clinical outcome. The translocation can be detected in (very few) of the lymphocytes in the peripheral blood of most healthy adults.

1.2 PATHOGENESIS

1.2.1 From the bone marrow to the germinal centre

The t(14;18), or, rarely, a biologically equivalent variant BCL-2 translocation such as t(2;18) or t(18;22) to the \(\kappa\) or \(\lambda\) light chain gene, is thought to be the first event in the ontogeny of follicular lymphoma, occurring in a progenitor pre-B-cell in the bone marrow. This cell develops normally and migrates to the germinal centre (follicle centre) of a lymph node. Normally, only 25% of germinal centre B-cells escape early death after antigenic selection. A cell with BCL-2 overexpression will be inappropri-
ately rescued from apoptosis, allowing it to survive and passively expand, resulting in an accumulation of long-lived t(14;18)+ B-cells.\textsuperscript{17} The malignant degeneration to follicular lymphoma occurs when a t(14;18)+ germinal centre B-cell acquires additional karyotypic or point mutations.\textsuperscript{17-19} Support for this hypothesis has been demonstrated in one patient who had two different follicular lymphomas (one $\kappa^+$ in the bone marrow and the other $\lambda^+$ in a lymph node) but with an identical BCL-2 recombination and identical variable, diversity and joining segments on the untranslocated IGH allele and thus derived from a common progenitor.\textsuperscript{20} The follicular lymphoma cells retain many of the features of normal germinal centre B-cells, such as class switch recombination and ongoing somatic hypermutation,\textsuperscript{19,21} and, also like their normal counterparts, they continue to communicate with neighbouring T cells and follicular dendritic cells (FDCs).\textsuperscript{22}

\subsection*{1.3 DIAGNOSIS}

\subsection*{1.3.1 Defining follicular lymphoma}
Because of its typical histology, follicular lymphoma is one of the few lymphomas that were recognised before the introduction of modern diagnostic tools (Figure 1-1). However, advances in haematopathology, immunology and genetics have incrementally led to greater reliability and reproducibility in the diagnostics of follicular lymphoma. The current 2008 definition in the fourth edition of the \textit{WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues} reads: “Follicular lymphoma is a neoplasm composed of follicle centre (germinal centre) B-cells (typically both centrocytes and centroblasts/large transformed cells), which usually has at least a partially follicular pattern. If diffuse areas of any size comprised predominantly or entirely of centroblastic cells are present in any case of follicular lymphoma, a diagnosis of diffuse large B-cell lymphoma is also made. Lymphomas composed of centrocytes and centroblasts with an entirely diffuse pattern in the sampled tissue may be included in this category [of follicular lymphoma].”\textsuperscript{13} Follicular lymphoma has a typical phenotype (CD5-, CD10+, CD19+, CD20+, BCL-2+ and BCL-6+) and, thus, cases which lack follicular architecture can be verified by demonstrating that or the t(14;18).\textsuperscript{13}

\subsection*{1.3.2 1, 2 and 3 (and 3A and 3B): The World Health Organization’s grades}
Follicular lymphoma has been graded in many previous lymphoma classifications (Rappaport, Working formulation, R. E. A. L.) according to the proportion of large cells (centroblasts).\textsuperscript{13} Also the current 2008 World Health Organization (WHO)
classification requires that follicular lymphomas are graded according to the proportion of centroblasts, expressed per ×40 high-power microscopic field (hpf; 0.159 mm²), in ≥10 randomly selected neoplastic follicles (Table 1-1; Figure 1-2). This method and the thresholds of five and 15 centroblasts are derived from Mann and Berard.23

Table 1-1: The 2008 WHO grading criteria.13

<table>
<thead>
<tr>
<th>Grade</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1</td>
<td>0–5 centroblasts per high-power field</td>
</tr>
<tr>
<td>2</td>
<td>6–15 centroblasts per high-power field</td>
</tr>
<tr>
<td>3</td>
<td>&gt;15 centroblasts per high-power field</td>
</tr>
<tr>
<td>3A</td>
<td>Centrocytes present</td>
</tr>
<tr>
<td>3B</td>
<td>Solid sheets of centroblasts or follicles entirely filled with centroblasts</td>
</tr>
</tbody>
</table>

The value of grading follicular lymphoma has been debated since the 1980s. Because the centroblasts of follicular lymphoma are cytologically identical to the centroblasts of DLBCL (note that “transformed cells” are used synonymously with centroblasts in the WHO definition above), it has been suspected that patients with more centroblasts have a worse prognosis and a more aggressive clinical course.

There have been numerous attempts to link centroblasts (or higher grades) with poor prognosis in follicular lymphoma.24-35 Some found associations between higher grade and worse outcome,24-26 many did not.27-35 There is no established consensus whether anthracyclines or autologous stem-cell transplantation produce cures in patients with higher-grade follicular lymphomas.26,29-42 Concerns over grading reproducibility between pathologists have also been voiced.43 Predictions based on increased proliferation, which is associated with higher grades, have also mostly been unfruitful.13,24,31,44-47 All agree that grade 1 and 2 share characteristics of indolent and incurable disease. A growing number of national and international lymphoma groups consider also grade 3A indolent and incurable, and patients with grade 3A are increa-
singly included in indolent lymphoma-trials, while others deem grade 3A aggressive and treat it with anthracycline-based regimens. The rare grade 3B, about 5% of all follicular lymphomas,\textsuperscript{13} is mostly treated as DLBCL. Grade 3B has much fewer cases of t(14;18)\textsuperscript{48,49} and a gene-expression distinct from that in both follicular lymphoma grade 1–3A and DLBCL.\textsuperscript{50} However, a statistically significant difference in clinical outcome between grade 3A and 3B patients has not been demonstrated.\textsuperscript{32-35}

1.3.3 Diffuse areas

Diffuse areas comprised predominantly of centrocytes are not thought to be clinically significant, although their proportion of the specimen should be noted according to the WHO.\textsuperscript{13} The presence of diffuse areas with more than 15 centroblasts/hpf in follicular lymphoma of any grade is equivalent to concomitant DLBCL, which is recorded as a separate diagnosis, and a known adverse risk factor.\textsuperscript{33} Only grade 1 and 2 can be diffuse; diffuse areas in grade 3 are by definition concomitant DLBCL.\textsuperscript{13}

1.4 EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS

Follicular lymphoma accounts for about 20% of all lymphomas, with the highest incidence in the USA and Western Europe\textsuperscript{13} (in Sweden, there are 200–230 new cases every year\textsuperscript{51}). It is much less common in other areas of the world, and it is twice as common in Caucasian as it is in African or Asian Americans.\textsuperscript{52} The disease incidence increases with age and peaks in quinquagenarians.\textsuperscript{13} In contrast to most other lymphomas, including DLBCL, the majority (58%) of the people follicular lymphoma afflicts are women.\textsuperscript{53} The disease is staged as other lymphomas with the system proposed at the Ann Arbor Conference in 1971.\textsuperscript{54} At diagnosis, most patients have both central and peripheral lymph node involvement and about two thirds of the patients have Ann Arbor stage III or IV and about 40% have bone-marrow involvement.\textsuperscript{53} The disease is occasionally primary in extranodal tissue, including skin, duodenum, ocular adnexa, breast and testis.\textsuperscript{13} Most patients do not have any of the Ann Arbor systemic (B) symptoms: otherwise unexplainable fever, night sweats or weight loss of more than 10%.

1.5 PROGNOSIS: THE CLINICAL COURSES OF FOLLICULAR LYMPHOMA

The prognosis of follicular lymphoma remained basically unchanged between 1970 and 2000, with a median overall survival time of about ten years,\textsuperscript{55,56} but with considerable individual variation, from less than one year to over 30 years.
1.5.1 Follicular lymphoma international prognostic index

Many clinical characteristics have been associated with prognosis in follicular lymphoma. Previously the international prognostic index constructed for DLBCL was applied. A follicular lymphoma-specific system, the follicular lymphoma international prognostic index (FLIPI) was developed, based on five clinical factors identified in retrospective analysis of clinical characteristics at diagnosis (Table 1-2).56 The FLIPI risk groups were equally sized and diverged with respect to overall survival. The fractions surviving at ten years in the three risk groups were, respectively, 71%, 51% and 36%.56 The FLIPI has been validated,57 also in different settings such as at first relapse58 and autologous stem cell transplantation.59,60 The FLIPI has recently been updated with FLIPI-2, as described below (1.8 Prognostic challenges).

Table 1-2: The FLIPI.56

<table>
<thead>
<tr>
<th>Factor</th>
<th>Score</th>
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<tbody>
<tr>
<td>Age over 60 years</td>
<td>0–1 factor: low risk</td>
</tr>
<tr>
<td>Ann Arbor stage III or IV</td>
<td>2 factors: intermediate risk</td>
</tr>
<tr>
<td>Haemoglobin less than 12 g/dL</td>
<td>3–5 factors: high risk</td>
</tr>
<tr>
<td>More than four nodal stations involved</td>
<td></td>
</tr>
<tr>
<td>Elevated serum lactate dehydrogenase</td>
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</table>

1.5.2 Transformation to diffuse large B-cell lymphoma

Transformation to DLBCL can occur at any time point and in any patient, being equally frequent in patients who previously have had an entirely asymptomatic disease as in others.61 In the 1940s transformation was identified as a substantial adverse event for patients with follicular lymphoma, being sometimes “present when the patient first consults the practitioner, in others it may never occur.” Transformation should preferably be verified with a biopsy, but in patients where this is impossible, a clinical diagnosis of transformation may be based on clinical signs such as a sudden rise in lactate dehydrogenase to twice the upper normal limit, rapid discordant localised lymph node growth, new involvement of unusual extranodal tissues (such as muscle, bone, brain and liver), new B symptoms or hypercalcaemia.62 In tumours that have transformed to DLBCL, there are additional genetic aberrations, almost always including p53 lesions.11 The number of cells in proliferation, detected as those positive for the proliferation marker Ki67, is always increased compared with the original follicular lymphoma.53 About 10%–30% of all follicular lymphomas will at some point transform, regardless of previous therapy; transformation renders a dismal prognosis with a historical median survival of less than two years.62,64 The annual transformation
incidence is reported to be 3%.\textsuperscript{62} In patients with transformation, attaining complete remission is paramount for survival.\textsuperscript{64} An additional translocation of MYC in the transformed specimen, that is, a “double hit” consisting of (the already present) BCL-2 [t(14;18)] and MYC [t(8;14), t(8;22) or t(8;9)], is associated with a particularly aggressive course.\textsuperscript{13}

\section*{1.6 THERAPY}

\subsection*{1.6.1 First-line therapy}

Since the early 1970s, patients with stage I and limited stage II disease receive local radiation of 30–35 Gy,\textsuperscript{65} achieving cure in some.\textsuperscript{66,67} However, patients with limited disease generally have good outcome, also with a wait-a-watch policy,\textsuperscript{68} and some of those could have been cured by the diagnostic surgical excision.\textsuperscript{6,69} Low-dose (4 Gy) radiotherapy may also be considered (even in advanced disease).\textsuperscript{70,71} Most patients have advanced disease at diagnosis. For those, a wait-and-watch policy till the emergence of symptoms or steady disease progression has been shown to be as good as mandatory treatment.\textsuperscript{72} Wait-and-watch is therefore the standard policy for patients with asymptomatic generalised disease. The disease can wax and wane and spontaneous regressions are seen in about 20\% of all patients.\textsuperscript{61} Features that prompt initiation of therapy include B symptoms, increasing or large tumour burden, cytopaenia due to bone marrow or spleen involvement, leukaemic progression, serous effusion and high lactate dehydrogenase levels. Interferon-\textit{α}\textsubscript{2a} (IFN-\textit{α}) has been shown to prolong the time before chemotherapy is needed.\textsuperscript{73} A univocal standard therapeutic option for patients with widespread follicular lymphoma does not exist. Many different chemotherapeutic regimens produce remissions of varying endurance, but the disease is still considered incurable, because it will relentlessly relapse. The most common first-line regimens in the 1990s were single-agent oral alkylators (chlorambucil or cyclophosphamide) and purine analogues (fludarabine), polychemotherapy such as cyclophosphamide-doxorubicin-vincristine-prednisone (CHOP) and cyclophosphamide-vincristine-prednisone (CVP).\textsuperscript{74-77} None of these was shown to be superior, although CHOP usually was more popular for patients with high-risk disease.\textsuperscript{78} IFN-\textit{α}, as an additive to chemotherapy and as maintenance after chemotherapy, was shown to prolong survival\textsuperscript{79} but did not become standard, because it was eclipsed by the monoclonal anti-CD20-antibody rituximab, introduced in the last years of the previous millennium. Rituximab was demonstrably effective in chemotherapy-refractory patients.\textsuperscript{80} Rituximab seems to add
benefit to any kind of conventional chemotherapy, as shown in rituximab combined with (R-)-CVP, R-CHOP, R-mitoxantrone-chlorambucil-prednisolone and R-CHOP + IFN-α.\textsuperscript{81-84} Rituximab is also effective as monotherapy,\textsuperscript{85-87} and when combined with IFN-α.\textsuperscript{88-90} There is no world-wide standard of care, and regional, national and continental treatment differences remain. According to Swedish guidelines, symptomatic previously untreated patients with low-risk FLIPI are given rituximab monotherapy (older patients can instead receive single-agent chlorambucil). Patients with high-risk FLIPI receive R-CHOP. However, new results have showed that rituximab combined with the East German nitrogen mustard bendamustine is superior to R-CHOP.\textsuperscript{91} It is likely that R-bendamustine will emerge as a new golden standard for treating follicular lymphoma with high-risk FLIPI. After induction therapy, rituximab maintenance for two years yields longer progression-free survival\textsuperscript{92} and is now part of standard therapy.

### 1.6.2 Relapse therapy

At localised relapse, a new biopsy should be obtained. If there is transformation to DLBCL, aggressive therapy is warranted. The prognosis is much worse than in de novo DLBCL.\textsuperscript{62} R-CHOP or a similar R-anthracycline-based regimen is standard in patients who have not previously received R-CHOP. Those previously treated with R-CHOP will receive salvage regimens based on rituximab combined with drugs such as cisplatin, carboplatin, etoposide, ifosphamide and high-dose cytarabine and methotrexate. Previously, a consolidating autologous stem cell transplantation was performed in those who were expected to tolerate the treatment, because a clear survival benefit was reported, with a median overall survival of about 50% at 5 years.\textsuperscript{93} After the introduction of rituximab, the place for stem cell transplantation has become less clear.\textsuperscript{94} A new algorithm for transformed disease recommends that previously untreated patients with local transformation should receive R-CHOP and local radiation as consolidation.\textsuperscript{95,96} However, autologous stem cell transplantations is an option always considered in patients with transformed follicular lymphoma.\textsuperscript{95} In patients who relapse without transformed disease, there is a wider range of treatment options. Treatment is only warranted if the disease is symptomatic, and wait-and-watch could be continued till the appearance of symptoms. Local relapse is treated with radiation. Systemic relapses that appear within three years after monotherapy (rituximab or chlorambucil) can be treated with R-CHOP, R-CVP, R-bendamustine or R-fludarabine-cyclophosphamide. A stem cell harvest at the end of treatment is recommended in younger patients. In relapses that appear after three years have passed since monotherapy, the previous
monotherapy can be repeated. Patients who quickly relapse after R-chemotherapy are
considered for autologous stem cell transplantation. In relapses more than three years
after R-chemotherapy, single rituximab or another variant of R-chemotherapy can be
given. After all sorts of relapse treatment, rituximab maintenance for two years is
recommended. Further relapses constitute an indication for autologous or allogeneic
stem cell transplantation. Allogeneic stem cell transplantation is probably curative, due
to the graft vs lymphoma effect, but the significant transplant-related mortality makes
this approach less feasible for many patients. A tandem approach of autologous
followed by allogeneic transplantation has shown encouraging results.

1.7 THE MICROENVIRONMENT

1.7.1 Immunodependence

Although having a constitutional BCL-2 overproduction, follicular lymphoma cells
depend on the microenvironment to escape apoptosis. Stromal cells, most
importantly the FDCs, are essential for the survival of both follicular lymphoma cells
and their normal counterparts, germinal centre B-cells. Like the germinal centre
B-cell, the follicular lymphoma cell is also supported and protected by interleukin (IL)-
4-producing CD4+ follicular B-helper T-cells (T_FH). IL-4 is the only cytokine that
is found in higher levels in follicular lymphoma than in reactive follicular hyperplastic
lymph nodes. It has been suggested that when follicular lymphoma migrates to new
sites of disease, it recruits cells that sustain it, such as FDCs and CD4+ T_FH. T_FH not
only produce IL-4 but also express CD40-ligand (CD40L; CD154). CD40L interacts
with CD40 on the malignant B cells and protects them from chemotherapy-induced
apoptosis. Furthermore, the tumour cells could be positively selected through their
antigen receptor, indicating that antigen stimulation may be involved in the growth,
progression and maybe transformation of follicular lymphoma. B–T cell inter-
actions through CD40–CD40L, maybe enhanced with concomitant antigen stimulation
through the B-cell receptor (BCR) of the malignant clone, protect against Fas receptor
(CD95)-transduced apoptosis. However, there is no tonic signalling of the BCR in
follicular lymphoma, and follicular lymphoma cells with impaired BCR signalling are
associated with poor prognosis and are more frequent at relapses.

1.7.2 Immunosurveillance

In 2004 a gene-expression analysis of follicular lymphoma patients showed that sur-
vival could be predicted from genes expressed in immune cells rather than in tumour
Genes expressed in macrophages correlated with poor prognosis, while genes expressed in T cells, especially in CD8+ (cytotoxic) T cells (CTLs), seemed associated with good prognosis. The association between CTLs and outcome hinted at some form of immunosurveillance that counteracted the lymphoma.

1.8 PROGNOSTIC CHALLENGES

Outcome in follicular lymphoma has improved since the introduction of rituximab, and the median overall survival is now at least 15 years.\textsuperscript{123} Still, the same prognostic problems remain since the 1940s: some patients die quickly, others remain asymptomatic and will never need treatment, while those who receive treatment respond differently to it. About 80% will receive treatment within a year from diagnosis, but half of the remaining 20% will never need treatment. At the other end of the spectrum are patients who relapse quickly (or never attain remission) after therapy, necessitating additional lines of therapy and transplantation. The underlying biology that drives the clinical heterogeneity cannot be identified by clinical prognostic indices. The prognostic accuracy of the FLIPI is less clear in patients receiving rituximab.\textsuperscript{124,125} The FLIPI-2, calculated on rituximab-treated patients, was therefore proposed in 2009 (\textbf{Table 1-3}),\textsuperscript{126} and it has already been verified\textsuperscript{127} but not replaced the FLIPI in clinical use. The FLIPI-2 was developed with respect to progression-free survival rather than overall survival, because the generally improved outcome for patients with follicular lymphoma has made overall survival an almost impossible endpoint. Furthermore, progression-free survival and related endpoints, such as to time to next treatment and time to treatment-failure, may be better for evaluating therapy and biological predictors, because overall survival will be affected by subsequent treatments. It is reasonable to believe that the prognostic qualities of the cells of the microenvironment are influenced by treatment used. It is also possible that prognostic properties of the tumour cells themselves change with therapy. Most prognostic studies of the microenvironment, and all studies of the WHO’s grading system, have been performed on patients treated before the introduction of rituximab.

\begin{table}[h]
\centering
\caption{The FLIPI-2.\textsuperscript{126}}
\begin{tabular}{|l|l|}
\hline
Factor & Score \\
\hline
Age over 60 years & 0 factor: low risk \\
Bone marrow involvement & 1–2 factors: intermediate risk \\
Haemoglobin less than 12 g/dL & 3–5 factors: high risk \\
Largest lymph node diameter greater than 6 cm & \\
Elevated beta-2 microglobulin & \\
\hline
\end{tabular}
\end{table}
2 AIMS

2.1 OVERALL AIM
To identify biological predictors for outcome in follicular lymphoma

2.2 SPECIFIC AIMS
To elucidate

1. the importance of T cells that coexist with the follicular lymphoma cells in the lymph nodes
2. the simultaneous impact of the entire immune microenvironment
3. the importance of local and systemic T cells in patients treated with rituximab
4. the WHO grading system for follicular lymphoma in patients treated with and without rituximab
3 PATIENTS AND METHODS

3.1 PATIENTS

3.1.1 The South Stockholm County cohort
All patients diagnosed with primary follicular lymphoma between January 1994 and January 2004 in South Stockholm County were identified at the Department of Pathology, Karolinska University Hospital, Huddinge. Lists of patients with the following diagnoses were assembled from archives: “Malignant lymphoma, not otherwise specified (M95903)”, “Malignant lymphoma, CB-CC, follicular (M96923)”, “Malignant lymphoma CB-CC (M96143)”, “B-cell lymphoma (M95933)”, “Malignant lymphoma, non-Hodgkin’s (M95913)” and “Malignant lymphoma, indolent (M95931)”. All pathology reports of these patients were examined, and all cases that could possibly be follicular lymphoma were identified. These cases were subject to a pathological review by Birgitta Sander and Birger Christensson, resulting in a cohort of 197 patients with reviewed diagnoses of follicular lymphoma, graded according to the current WHO criteria. Clinical data were gathered from patient files from Karolinska University (n=166), Stockholm South (n=23), S:t Görans (n=2), Södertälje (n=1) and Visby (n=5) hospitals. Because the pathology department is the only one in the region, the identified cohort is unselected and population-based. The 197 patients were treated according to local practice. This cohort was used in papers I, II and IV.

3.1.2 The Norwegian Radium Hospital cohort
These patients were diagnosed between January 1994 and January 2004 and identified in the patient database of the Norwegian Radium Hospital. The database includes information on clinical characteristics, treatment and follow-up. A subsequent review (by Birgitta Sander) of all available specimens resulted in a cohort of 317 patients with verified follicular lymphoma, graded according to the current WHO criteria. The patients were treated according to local practice. This cohort was used in paper IV.

3.1.3 The Nordic Lymphoma Group’s rituximab trials
Since the late 1990s, the Nordic Lymphoma Group (NLG) has undertaken two randomised trials for indolent lymphoma, M3903590 (phase II, accrual 1998–1999) and ML16865 (phase III, accrual 2002–2008). In both trials, all patients received rituximab
and were 1:1 randomised to the addition of IFN-α priming. Inclusion criteria were symptomatic, advanced indolent CD20+ lymphoma, previously untreated or in first relapse after a previous response to only oral alkylators or local radiation. The previous treatment had been completed at least six months before inclusion. The two trials had similar outlines (Figure 3-1).

Treatment was given in two consecutive cycles, each consisting of four doses of rituximab (375 mg/m²) with or without IFN-α. In M39035, patients with partial or minor response (for definition, see below) at the response evaluation after the first cycle (EV-1), were randomised to receive the second cycle with or without IFN-α. In ML16865, IFN-α was randomised upfront. In both studies, patients with stable or progressive disease at EV-1 were not eligible for the second cycle, and left the trials for off-study salvage therapy. After the second cycle of therapy, a new response evaluation (EV-2) was performed. The response categories were defined according to the 1999 Cheson criteria:128 complete (CR), complete/unconfirmed (CRu), and partial (PR) response, and stable (SD) and progressive (PD) disease. An additional subcategory of SD, minor response (MR), was used only to allow patients at EV-1 to be randomised to cycle 2. MR was defined as a decrease in the sum of the products of the greatest diameters in all
measurable lesions of at least 25% but less than 50% from baseline and/or improvement of disease symptoms. In M39035 126 (92 follicular lymphoma) patients and in ML16865 313 (259 follicular lymphoma) patients were included, in total 439 (351 follicular lymphoma) patients. Christer Sundström, Uppsala, reviewed all diagnostic specimens in the two trials. Information on these 351 follicular lymphoma patients was obtained from the trial databases and used in papers III and IV.

3.2 METHODS

3.2.1 Flow cytometry analysis

At Karolinska Huddinge, flow cytometry is a routine procedure in the lymphoma diagnostic work-up. In the South Stockholm County cohort, 139 grade 1–3A patients without concomitant DLBCL had had primary diagnostic lymph node cells examined with flow cytometry, using three-colour fluorescence according to standard procedures. Briefly, suspended cells from biopsies were washed before mixing with appropriate concentrations of fluorochrome-conjugated monoclonal antibodies to B-cell antigens, such as CD19, CD20, CD22, CD23, CD10 and immunoglobulin κ and λ chains. T-cell markers analysed were CD2, CD3, CD4, CD5, CD7 and CD8. In addition, cells were analysed for CD45, CD14, CD52, HLADR, CD16, CD56 and CD25. All antibodies were obtained from Becton Dickinson (Mountain View, CA). After incubation for 30 minutes at room temperature, cells were washed with phosphate buffered saline. For data acquisition and analysis, a FACSCalibur (Becton Dickinson) was used with Cell Quest software (Becton Dickinson). All samples were analysed by setting appropriate side and forward scatter gates around the mononuclear cell population using CD45/CD14 for gate setting. Consistency of analysis parameters was ascertained by calibrating the flow cytometer with calibrating beads and FacsComp software, both from Becton Dickinson. We performed a central review of all flow cytometry plots from the primary diagnostic tissue (mostly lymph nodes) and the data were utilised in papers I and II. In the NLG trials flow cytometry of blood and lymph nodes was performed according to a standardised protocol that mandated analysis of the following antigens: CD19, CD20, CD23, CD10, CD3, CD4, CD5, CD8, κ, λ and (in blood only) CD16/CD56. Results from these analyses were obtained from the trial databases and validated using the original flow cytometry reports, requested from the different participating trial centres and used in paper III (N=250). All flow results in this thesis were reported as percents of cells within mononuclear gate positive for each antibody (Figure 3-2).
3.2.2 Microscopy and immunohistochemistry

Biopsies of all patients participating in this work have been centrally reviewed for diagnosis and graded according to the WHO criteria (N=828). The grading data constituted the basis for paper IV. Routine immunohistochemistry stainings were also assessed and utilised in papers I and IV. Study-specific immunostainings were performed on the tissue microarray for the computerised image analysis of paper II (described below).

3.2.3 Tissue microarray

The source population for tissue microarray was all patients in the South Stockholm County follicular lymphoma cohort with sufficient diagnostic material for tissue microarray analysis but not grade 3B disease or concomitant DLBCL. We selected two extreme-outcome groups. The poor-outcome group contained all patients who died from lymphoma-related causes within five years from diagnosis. The causes of death were obtained from patient files, amended with data from the National Causes of Death Register in four uncertain cases. For inclusion in the good-outcome group, patients first had to fulfill two general criteria: no possible lymphoma-related death and no autologous or allogeneic transplantation. Furthermore, for the good-outcome patients, one of the following three statements had to be true: (1) never treated against lymphoma
and followed for at least five years (n = 11), (2) never relapsed after first-line anti-lymphoma treatment and followed for at least eight years (n = 14) or (3) relapsed but never received frequent (at least three years between) treatments and followed for at least ten years (n = 12). These selection criteria rendered 37 and 33 patients in the good- and poor-outcome group, respectively. All 70 patients were identified and grouped before the construction of the tissue microarray. For the array, twin 1.2 mm cores were taken from the diagnostic and relapse tumour biopsies of the patients and put into a paraffin-embedded tissue microarray. One good and two poor diagnostic cases had insufficient material left for successful core production, leaving 67 patients. The tissue microarray was analysed in paper II, using computerised image analysis.

3.2.4 Computerised image analysis

After immunostaining slides from the above-described tissue microarray of the 67 extreme-outcome patients, cell populations were quantified using an automated scanning microscope and computerised image analysis system (Ariol SL-50; Genetix) under the supervision of an expert haematologic-pathologic team (Santiago Montes-Moreno and the respondent of this thesis). The system allows for precise demarcation by drawing boundaries on the virtual slides and the follicular and interfollicular areas within each core were defined (Figure 3-3:A). Stainings against the nonnuclear antigens CD3, CD7, CD8, CD56, CD57, CD68, granzyme B, TIA-1, perforin and tryptase were strong and made individual cells difficult to discern by the Ariol system, due to overlapping positivity, why these subsets were quantified as the fraction of cellular antibody-positive area divided by total cellular area (Figure 3-3:B1-C1). Total cellular area was calculated as the sum of the antibody-positive and antibody-negative cellular areas. Cells positive for the nuclear marker forkhead box protein 3 (FOXP3) and the weak CD4 and programmed death-1 (PD-1; CD279) stainings were better quantified as numbers of positive cells divided by total cellular area (Figure 3-3:B2-C2). The software algorithm was determined for each marker and applied in all the samples in the same way. For all measurements, validation of the specificity of the staining was done by a haematopathologist (Santiago Montes-Moreno). Also, to ensure the robustness of the results, the automated quantification results were verified using flow cytometry results from the same specimens. The follicular and interfollicular areas in each core were separately quantified, to get results in three different compartments (total core, follicular and interfollicular) for each antibody in each case, except cases with entirely diffuse cores, where only total core values were extracted.
Figure 3-3: Computerised image analysis of tissue microarrays. Panel A shows CD3 stainings in a part of a tissue microarray slide. Follicular and interfollicular areas are defined; one case is diffuse. Panel B shows close-up examples of non-nuclear CD8 (B1) and nuclear FOXP3 (B2) stainings. Panel C shows the computerised interpretation of these stainings, with CD8 positivity (C1) quantified as the percent of positive (red) area divided by the total cellular area (positive and negative [green] areas together) and with FOXP3 positivity (C2) quantified as the number of positive cells (red dots) divided by total cellular area.

3.2.5 Statistics

In papers I, III and IV predictors were analysed with respect to time to event (survival analysis), using Kaplan–Meier curves\(^{129}\) and the log-rank test,\(^{130}\) and Cox regression\(^{131}\) for multivariate analysis. In papers II and III predictors were analysed with respect to dichotomous (paper II) and ordinal (paper III) outcome variables, using Wilcoxon–Mann–Whitney, Kruskal–Wallis or Spearman tests, depending on the nature of the variables, and logistic regression for multivariate analysis. All \(P\)-values are two-tailed.
4 RESULTS

4.1 PAPER I: CD8+ T-CELL LEVELS IN THE MICROENVIRONMENT PREDICT SURVIVAL

Flow cytometry assays were performed on diagnostic lymph nodes in 139 grade 1–3A patients diagnosed with primary follicular lymphoma at Karolinska Huddinge between 1994 and 2004. Table 4-1 shows the levels of the lymphocyte subsets and their associations with overall survival.

**Table 4-1: Lymphocyte subset levels in paper I.**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Median</th>
<th>Min</th>
<th>p25</th>
<th>p75</th>
<th>Max</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ (B-cells)</td>
<td>67%</td>
<td>15%</td>
<td>54%</td>
<td>77%</td>
<td>94%</td>
<td>1.02 (1.00–1.04)</td>
</tr>
<tr>
<td>Clonal CD19+</td>
<td>58%</td>
<td>6%</td>
<td>44%</td>
<td>69%</td>
<td>94%</td>
<td>1.00 (0.99–1.02)</td>
</tr>
<tr>
<td>Nonclonal CD19+</td>
<td>2%</td>
<td>0%</td>
<td>1%</td>
<td>6%</td>
<td>40%</td>
<td>1.00 (0.95–1.06)</td>
</tr>
<tr>
<td>CD3+ (T cells)</td>
<td>31%</td>
<td>7%</td>
<td>22%</td>
<td>44%</td>
<td>85%</td>
<td>0.99 (0.96–1.02)</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>24%</td>
<td>4%</td>
<td>17%</td>
<td>35%</td>
<td>69%</td>
<td>0.98 (0.96–1.01)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>6%</td>
<td>1%</td>
<td>4%</td>
<td>9%</td>
<td>26%</td>
<td>0.90 (0.82–0.98)</td>
</tr>
<tr>
<td>CD4/CD3 ratio</td>
<td>0.78</td>
<td>0.26</td>
<td>0.72</td>
<td>0.84</td>
<td>1.16</td>
<td>6.75 (0.51–89.9)</td>
</tr>
<tr>
<td>CD8/CD3 ratio</td>
<td>0.20</td>
<td>0.06</td>
<td>0.15</td>
<td>0.26</td>
<td>0.48</td>
<td>0.06 (0.00–2.78)</td>
</tr>
</tbody>
</table>

HR denotes hazard ratio and CI confidence interval (with respect to overall survival and adjusted for the FLIPI).

Clonal cells were defined as the highest of CD19+κ+ or CD19+λ+ and nonclonal cells as the lowest of CD19+κ+ or CD19+λ+.

Several clinical factors also correlated with survival. These competed with flow cytometry results in multivariate analysis, shown in Table 4-2.

**Table 4-2: Multivariate models in paper I.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Overall survival</th>
<th>Disease-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (year)</td>
<td>1.08</td>
<td>1.05–1.11</td>
</tr>
<tr>
<td>Male sex</td>
<td>3.58</td>
<td>1.83–7.03</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>0.74</td>
<td>0.61–0.91</td>
</tr>
<tr>
<td>LDH (multiples of UNL)</td>
<td>3.86</td>
<td>1.58–9.43</td>
</tr>
<tr>
<td>Stage III–IV</td>
<td>3.66</td>
<td>1.43–9.37</td>
</tr>
<tr>
<td>≥2 extranodal sites</td>
<td>5.69</td>
<td>2.36–13.75</td>
</tr>
<tr>
<td>Dementia</td>
<td>14.98</td>
<td>2.65–84.58</td>
</tr>
<tr>
<td>CD8+ T cells (%)</td>
<td>0.86</td>
<td>0.78–0.94</td>
</tr>
</tbody>
</table>

HR denotes hazard ratio, CI confidence interval, LDH lactate dehydrogenase and UNL upper normal limit.

Higher nodal CD8+ CTL content was independently associated with better overall and disease-specific survival. Moreover, high CTL levels correlated with less need for early treatment, but not with any important clinical characteristics.
4.2 PAPER II: A UNIFYING MICROENVIRONMENT MODEL

Our hypothesis, based on previous findings, was that several immune cell subsets are important for disease outcome and that their individual importance should be demonstrable in the same analysis and in competition with clinical factors. Specifically, we hypothesised that (1) CD8+ CTLs are associated with good prognosis, as are (2) T cells positive for PD-1 and FOXP3, while (3) CD4+ helper T cells are associated with poor prognosis. Quantifications of immunostained tissue microarrays were performed on diagnostic and relapse biopsies of 67 extreme-outcome patients as described above.

The computerised image analysis quantifications were verified using corresponding flow cytometry results obtained from the same samples. There were strong linear associations between the computerised image and flow cytometry results in all evaluable subsets (CD3, CD4, CD7, CD8 and FOXP3/CD3+CD25+). The only nonmalignant cell type more frequent inside than outside follicles were the PD-1+ T cells.

Several subsets quantified by image analysis were marginally significant or stronger in univariate analysis (CD4, CD8, CD56, CD57, CD68, FOXP3, PD-1, TIA-1, granzyme B and perforin). These competed in a multivariate analysis in which only total-core values were used, because we did not want to lose information from the diffuse cases that did not have follicular or interfollicular compartments (Table 4-3:A, Total core model). In a second multivariate analysis (Table 4-3:B, Compartmental model), subsets in the follicular and interfollicular compartments were analysed. Both multivariate analyses were adjusted for the FLIPI.

### Table 4-3: Multivariate models in paper II.

<table>
<thead>
<tr>
<th>Model</th>
<th>Factor</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A, Total core model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 total core</td>
<td>1.26</td>
<td>1.03–1.54</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>PD-1 total core</td>
<td>0.58</td>
<td>0.37–0.92</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>CD8 total core</td>
<td>0.94</td>
<td>0.89–0.99</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td><strong>B, Compartmental model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 follicular</td>
<td>2.16</td>
<td>1.21–3.87</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>FOXP3 follicular</td>
<td>0.09</td>
<td>0.01–0.66</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>PD-1 follicular</td>
<td>0.34</td>
<td>0.13–0.84</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>CD8 interfollicular</td>
<td>0.86</td>
<td>0.76–0.97</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>CD68 interfollicular</td>
<td>1.36</td>
<td>1.01–1.82</td>
<td>0.040</td>
<td></td>
</tr>
</tbody>
</table>

OR denotes odds ratio and CI confidence interval (adjusted for the FLIPI).
Figure 4-1: Panel A shows disease-specific survival according to the CD4/CD8 ratio (left), follicular/interfollicular CD4 ratio (middle) and both combined (right). Panel B shows examples of good (1–4) and poor (5–8) outcome. Case 1 is CD8-dominated. Case 2 is rich in CD8, FOXP3 and CD68. Case 3 is PD-1-dominated (but low in CD57). Case 4 is high in both CD8 and PD-1 (and CD57). Cases 5–7 contain few CD8 and many CD4; case 7 is also rich in PD-1 and CD68. Case 8 is CD68-dominated. Patients’ histories are summarised accordingly: age and sex, FLIPI risk group (L)ow, (I)ntermediate or (H)igh. First-line treatment, response. (R)elapse number (and if [T]ransformed): treatment, response. Survival status, follow-up time.
Higher CD4/CD8 ratios in all compartments correlated with poor outcome, as did higher ratios between follicular and interfollicular CD4+ cells. These two ratios, each and together, were the best dichotomous predictors of outcome (Figure 4-1:A). Figure 4-1:B shows examples of the most important immunostainings.

There were no significant differences in subsets or ratios between the diagnostic and the subsequent biopsies when both were follicular lymphoma (n=13). In four subsequent biopsies, the disease had transformed to DLBCL, with an altered microenvironment: all nonmalignant subsets except macrophages were scarcer in the transformed specimen, especially CD4+ cells, probably due to the absence of recruiting follicle centres. There was, accordingly, also a tendency towards lowered CD4/CD8 ratios.

### 4.3 PAPER III: RITUXIMAB, IFN-α AND T CELLS IN LYMPH NODES AND IN BLOOD

Many of the clinicobiological effects of rituximab are probably dependent on T cells of both the CD4 and CD8 lineage. We therefore hypothesised that high pre-treatment levels of both CD4+ and CD8+ cells in the tumour microenvironment as well as in the blood are advantageous for the response to rituximab. IFN-α, another drug used in follicular lymphoma, alters and potentiates immune cells that react against the rituximab-binding CD20+ lymphoma. Using flow cytometry, we evaluated the T cells in tumours and/or blood in a total of 250 follicular lymphoma patients included in the above-described two NLG trials that compared single rituximab with IFN-α-rituximab combinations.

**Table 4-4: Lymphocyte subset levels in paper III.**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Median</th>
<th>p25</th>
<th>p75</th>
<th>OR</th>
<th>P</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour-CD3</td>
<td>29%</td>
<td>18%</td>
<td>40%</td>
<td>0.7</td>
<td>&lt;0.001</td>
<td>0.8</td>
<td>0.027</td>
</tr>
<tr>
<td>Tumour-CD4</td>
<td>21%</td>
<td>15%</td>
<td>32%</td>
<td>0.7</td>
<td>0.005</td>
<td>0.8</td>
<td>0.18</td>
</tr>
<tr>
<td>Tumour-CD8</td>
<td>6%</td>
<td>4%</td>
<td>10%</td>
<td>0.7</td>
<td>0.027</td>
<td>0.5</td>
<td>0.041</td>
</tr>
<tr>
<td>Tumour-CD19</td>
<td>67%</td>
<td>50%</td>
<td>77%</td>
<td>0.7</td>
<td>0.001</td>
<td>1.2</td>
<td>0.17</td>
</tr>
<tr>
<td>Blood-CD3</td>
<td>56%</td>
<td>29%</td>
<td>69%</td>
<td>0.7</td>
<td>0.99</td>
<td>0.8</td>
<td>0.034</td>
</tr>
<tr>
<td>Blood-CD4</td>
<td>30%</td>
<td>17%</td>
<td>41%</td>
<td>0.7</td>
<td>0.51</td>
<td>0.7</td>
<td>0.003</td>
</tr>
<tr>
<td>Blood-CD8</td>
<td>23%</td>
<td>14%</td>
<td>35%</td>
<td>0.7</td>
<td>0.66</td>
<td>0.7</td>
<td>0.17</td>
</tr>
<tr>
<td>Blood total lymphocytes (cells/ml)</td>
<td>1.3</td>
<td>0.9</td>
<td>1.9</td>
<td>0.74</td>
<td>1.0</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

OR denotes odds ratio. For the odds ratio, the units of the lymphocyte subsets are increments of ten percentage points.
Figure 4-2: Lymphocytes' associations with treatment responses stratified by randomisation to IFN-α.
Higher levels of CD3+, CD4+ and CD8+ T cells in both tumours and blood correlated with superior treatment responses. All tumour T-cells were significant at EV-1 and EV-2, except CD4+ T cells at EV-2, while blood (CD3+ and CD4+) T-cells were only significant at EV-2 (Table 4-4). In multivariate analysis tumour-CD3+ and blood-CD4+ cells were independent (Table 4-5). CD4+ cells were favourable regardless of treatment arm, but CD8+ cells only in patients treated with single rituximab (Figure 4-2), because IFN-α improved responses especially in patients with low CD8+ cells.

Table 4-5: Multivariate models in paper III.

<table>
<thead>
<tr>
<th>Model</th>
<th>Factor</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, Response at EV-1</td>
<td>Tumour-CD3</td>
<td>0.7</td>
<td>0.6–0.9</td>
<td>0.003</td>
</tr>
<tr>
<td>B, Response at EV-2</td>
<td>Tumour-CD3</td>
<td>0.5</td>
<td>0.3–0.9</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Blood-CD4</td>
<td>0.6</td>
<td>0.4–0.9</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>Age &gt;60 years</td>
<td>13.0</td>
<td>3.0–58.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

OR denotes odds ratio and CI confidence interval.
For the odds ratio, the units of the lymphocyte subsets are increments of ten percentage points.

Higher levels of blood-CD3+ (P=0.003) and blood-CD4+ (P=0.046) cells predicted longer overall survival and higher levels of blood-CD8+ cells (P=0.046) longer times to next treatment (Figure 4-3). Furthermore, higher CD3+, CD4+ and CD8+ levels in blood, but not in tumours, correlated strongly with good FLIPI factors (normal lactate dehydrogenase, ≤4 involved nodal areas and normal haemoglobin). Blood-CD3 were an independent predictor in multivariate overall survival analysis (P=0.008).

Figure 4-3: Panel A shows overall survival according to blood-CD3+ T-cell levels and panel B time to next treatment according to blood-CD8+ T-cell levels.
4.4 PAPER IV: GRADING FOLLICULAR LYMPHOMA IN THE RITUXIMAB ERA

The aim of this study was to evaluate the 2008 WHO grades in a large series of patients of whom 40% were upfront rituximab-treated. We had three major questions: (1) Is grade 3B a disease clinically more similar to DLBCL (aggressive but curable with anthracyclines)? (2) Do patients with grade 3A have similar outcome as those with grade 1–2 (indolent and incurable with anthracyclines)? (3) What is the predictive value of the WHO grades in patients treated upfront with rituximab?

First, we reviewed 514 consecutive follicular lymphoma cases (197 at the Karolinska University Hospital at Huddinge and 317 at the Norwegian Radium Hospital), resulting in a population-based cohort with long follow-up times (median 9.9 years [range, 4.6–16.0]), designated cohort A. From the NLG’s rituximab trials an independent cohort B of 314 patients was constructed (subtracting 37 patients who were part of cohort A). Of this total of 828 patients, 43 in cohort A were excluded because of concomitant DLBCL, rendering 785 analysed for outcome. Compared with grades 1, 2 and 3A, grade 3B was rapidly lethal, but outcome was improved after upfront anthracycline-containing therapy (Figure 4-4). Patients with grade 3B suffered no relapses or deaths beyond five years of follow-up, indicating an aggressive but curable disease.

Figure 4-4: Panel A shows overall survival according to WHO grades. Thirty-one patients whose biopsies did not allow for distinction between grades 1 and 2 are not shown in the graph. Panel B shows overall survival according to grades and stratified by upfront anthracycline-containing treatment; those who received upfront rituximab were excluded from the analysis.
Grade 3B was independent in multivariate analysis (Table 4-6), as was the benefit from upfront anthracyclines in grade 3B patients ($P=0.012$).

Table 4-6: Multivariate model with respect to overall survival in all 785 patients.

<table>
<thead>
<tr>
<th>Factor</th>
<th>HR</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;60 years</td>
<td>2.7</td>
<td>2.1–3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>1.7</td>
<td>1.2–2.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Haemoglobin &lt;12 g/dl</td>
<td>1.8</td>
<td>1.3–2.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Bone marrow involvement</td>
<td>1.8</td>
<td>1.4–2.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B symptoms</td>
<td>1.5</td>
<td>1.1–2.0</td>
<td>0.019</td>
</tr>
<tr>
<td>Grade 3B</td>
<td>2.4</td>
<td>1.3–4.2</td>
<td>0.003</td>
</tr>
</tbody>
</table>

HR denotes hazard ratio, CI confidence interval and LDH lactate dehydrogenase.

Grade 3B patients were also different in their clinical characteristics. In the population-based cohort A, only 34% of grade 3B patients were female compared with 49% of grade 1–3A patients, and grade 3B patients also had less bone-marrow (17% vs 40%) and stage III–IV (41% vs 66%) disease. As expected, there was much more Ki67-positivity in grade 3B than in grade 1–3A specimens ($P<0.001$). Furthermore, positivity for BCL-2 differed significantly between grade 3B (76%) and 1–3A (93%) biopsies ($P=0.020$; $n=142$), as did positivity for p53 (40% vs 4%; $P<0.001$; $n=122$), but not that for BCL-6 or CD10.

Patients with grade 3A had a similar clinical course as those with grade 1–2 and did not show any benefit from upfront anthracycline-containing therapy (Figure 4-4). However, increasing grades of 1, 2 and 3A were associated with better overall survival and time to treatment-failure in patients treated upfront with rituximab (Figure 4-5).

![Figure 4-5: Outcome according to grades 1, 2 and 3A in patients treated upfront with rituximab. Panel A shows overall survival and panel B time to treatment-failure in patients given upfront rituximab-containing chemotherapy.](image-url)
Table 4-7 shows that with upfront rituximab-containing therapy, higher grades of 1, 2 and 3A were significant in multivariate analysis, also in both types of upfront rituximab-containing regimens (without or with chemotherapy).

**Table 4-7: Multivariate analysis of grade 1, 2 and 3A in patients given upfront rituximab.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Patients</th>
<th>Overall survival</th>
<th>Time to treatment-failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>HR</td>
</tr>
<tr>
<td>Rituximab-containing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>130</td>
<td>42.5</td>
<td>0.007</td>
</tr>
<tr>
<td>Grade 2</td>
<td>149</td>
<td>48.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Grade 3A</td>
<td>27</td>
<td>8.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Rituximab without chemotherapy*</td>
<td>279</td>
<td>100</td>
<td>0.021</td>
</tr>
<tr>
<td>Grade 1</td>
<td>122</td>
<td>43.7</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>140</td>
<td>50.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Grade 3A</td>
<td>17</td>
<td>6.1</td>
<td>‡</td>
</tr>
<tr>
<td>Rituximab and chemotherapy†</td>
<td>27</td>
<td>100</td>
<td>0.076</td>
</tr>
<tr>
<td>Grade 1</td>
<td>8</td>
<td>29.6</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>9</td>
<td>33.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Grade 3A</td>
<td>10</td>
<td>37.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

HR denotes hazard ratio and CI confidence interval.
* All patients in this category were participants in the NLG randomised trials.
† 26/27 patients received R-CHOP; 1/27 received R-CVP.
‡ Not calculable, because there were no deaths in the grade 3A category.

Conversely, in patients treated upfront with alkylators, increasing grades correlated with inferior overall survival ($P=0.025$).

We also examined the numbers of Ki67-positive cells and of centroblasts/hpf. These proliferative markers did not correlate with outcome, although they showed trends similar to the grades in the subgroups of patients treated upfront with rituximab and alkylators.
5 DISCUSSION

5.1 THE MICROENVIRONMENT BEFORE RITUXIMAB (PAPERS I AND II)

Follicular lymphoma is a disease within the immune system, and it has long been suspected that the follicular lymphoma tumour cells are dependent on nonmalignant immune and stromal cells to survive.\textsuperscript{100-110,113} Still, the search for biological prognostic markers used to be focused on follicular lymphoma cells rather than their surrounding immune cells. In 2004 Dave et al. reported—to their surprise—that genes expressed in nonmalignant tumour-infiltrating T cells and macrophages had stronger bearing on survival than genes expressed in the tumour cells themselves.\textsuperscript{122} In the same year our group also showed that high levels of CD25+ and CD8+ T cells were associated with better outcome.\textsuperscript{133} That study was expanded into the first paper of the present thesis, demonstrating that increasing lymph node content of CD8+ CTLs were independent (Table 4-2).\textsuperscript{134} Several other studies of the microenvironment of follicular lymphoma were performed around the same time (Table 5-1).


<table>
<thead>
<tr>
<th>First author of report(s)</th>
<th>Therapy</th>
<th>Macrophages</th>
<th>T cells (CD3+)</th>
<th>CD4+ Helper T</th>
<th>CD8+ CTLs</th>
<th>FOXP3+ Tregs</th>
<th>PD-1+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farinha\textsuperscript{135,136}</td>
<td>BP-VACOP</td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>(a)N; (d)G; (f/p)P</td>
<td>-</td>
</tr>
<tr>
<td>Carreras\textsuperscript{137,138}</td>
<td>Various</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>(a)G; (l)N; (f)G</td>
<td>(a)G</td>
</tr>
<tr>
<td>Lee\textsuperscript{139}</td>
<td>Various</td>
<td>N</td>
<td>CD7+ N</td>
<td>G</td>
<td>N</td>
<td>(p)G</td>
<td>-</td>
</tr>
<tr>
<td>Alvaro\textsuperscript{140,141}</td>
<td>Various</td>
<td>N; STAT1+ P</td>
<td>N</td>
<td>N</td>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wahlin (Paper I)\textsuperscript{134}</td>
<td>Various</td>
<td>-</td>
<td>G</td>
<td>N</td>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glas\textsuperscript{142}</td>
<td>Various</td>
<td>N</td>
<td>N</td>
<td>(f)P</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Klapper\textsuperscript{31}</td>
<td>CHOP MCP</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kelley\textsuperscript{143}</td>
<td>Various</td>
<td>(f)P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(f)P</td>
<td>-</td>
</tr>
<tr>
<td>Tzankov\textsuperscript{144}</td>
<td>Various</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Byers\textsuperscript{145}</td>
<td>Various</td>
<td>P</td>
<td>G;CD7+ G</td>
<td>P</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>de Jong\textsuperscript{146}</td>
<td>CVP Fludarabine</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>(f)G; (f/N)</td>
<td>-</td>
</tr>
<tr>
<td>Wahlin (Paper II)\textsuperscript{147}</td>
<td>Various</td>
<td>(f)P</td>
<td>N</td>
<td>(a)P; (f)P</td>
<td>(a)G; (l)G</td>
<td>(c)G; (l)G</td>
<td>(a)G</td>
</tr>
</tbody>
</table>

BP-VACOP denotes bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine and prednisone. STAT signal transducers and activators of transcription and MCP mitoxantrone-chorambucil-prednisolone. Association with outcome is denoted as P (poor), G (good) and N (none or not significant). Distributions of cells are indicated as follows: (f) denotes follicular, (p) perifollicular, (i) interfollicular, (d) diffuse and (a) any pattern of distribution. Underlined findings were significant in multivariate analysis.

Table 5-1 allows one to discern a couple of general trends. Prior to our paper II, macrophages (defined as CD68+ cells) were sometimes associated with poor\textsuperscript{135,141,143,145} but
never with good outcome. T cells (positive for the pan-T markers CD3 or CD7) and particularly CD8+ CTLs were sometimes associated with good but never with poor outcome. With regard to the CD4+ T cells the picture is more contradictory and complex. A high number of CD4+ T cells correlated with good outcome in one study but with poor outcome in another. A predominantly follicular distribution of CD4+ T cells was associated with early transformation in a third report. The most frequent helper T subset in lymphatic tissue, especially inside follicles, is TFH. However, two scarce but also CD4+ T-cell subsets, regulatory T cells (Tregs; defined by positivity for FOXP3 and usually strongly CD25+) and PD-1+ T cells are found at low frequencies in follicular lymphoma nodes (PD-1+ cells preferentially inside the follicles). FOXP3+ Tregs were mostly associated with good but sometimes with poor prognosis, and the results were contradictory with respect to the importance of their infiltration pattern (Table 5-1). PD-1+ T cells were associated with good prognosis in a study published during the analysis-phase of paper II.

Paper II presents a successful attempt to unify these previous results, showing five sub-sets to be independently predictive. CD8+ CTLs, PD-1+ T cells and FOXP3+ Tregs correlated with good but (particularly follicular) CD4+ helper T cells and interfollicular macrophages with poor outcome (Table 4-3; Figure 4-1). This was the first demonstration of a simultaneous impact of a multitude of microenvironmental components, showing that prognosis in follicular lymphoma is affected by several factors in the microenvironment, rather than being dictated by an individual immune cell subset. We believe that our methodology was instrumental for this multifactorial model-building. Firstly, the study contained clinical extreme-outcome patients, not only with regard to chronological follow-up time and mortality, but also to disease-related clinical characteristics, disease aggressiveness and need for treatment. Selections based only on survival status and follow-up times would probably have obscured the microenvironmental factors, because such a selection would have been more influenced by differences unrelated to lymphoma biology, such as patients’ abilities to tolerate intensive treatment and their age. These extreme contrasts in clinical outcome presumably also presented large microenvironmental differences, which thus would be easier to detect than in an unselected cohort. Secondly, our unique method of computerised image analysis of the follicular and interfollicular compartments allowed for very precise quantifications, which, in contrast to manual semi-quantifications, were lossless, unbiased and
reproducible. Moreover, the quantifications were validated with flow cytometry results from the same specimens.

The findings in paper II confirmed that CD8+ CTLs correlate with good prognosis, regardless of their distribution patterns in the follicular lymphoma nodes, although the vast majority of CTLs are interfollicular. Markers of activated cytotoxicity in CTLs had diverging results and did not hold significance in multivariate analysis. We suggest that CTLs are beneficial because of their secretion of IFN-γ. IFN-γ overrules the growth-promoting interaction between stromal cells and follicular lymphoma cells and inhibits B-cell migration, plausibly preventing promotion and dissemination of the disease. IFN-γ also favourably modulates the development of naïve CD4+ T cells, which differentiate to either helper1 (T_{H1}) or helper2 (T_{H2}) T cells, and the T_{H2} further differentiate into T_{FH}. IFN-γ tips the balance towards T_{H1} and away from T_{H2}–T_{FH}. The T_{H1} are crucial for cell-mediated immunity and they also produce IFN-γ as well as IL-2, required for function and proliferation of CTLs, while the T_{H2} specialise in B-cell activation. The T_{FH} are distinguished by several criteria including chemokine receptor expression (C-X-C chemokine receptor type 5 [CXCR5]), location (B-cell follicles) and function (even more dedicated to B-cell help). The T_{H2} and T_{FH} counteract IFN-γ by producing IL-4 that drives the CD4+ T-cell differentiation towards their own phenotypes and inhibits the T_{H1} and CTLs. This balance between T_{H1}–CTL cytokines on one hand and T_{H2}–T_{FH} cytokines on the other (the IFN-γ/IL-4 ratio) is skewed in follicular lymphoma: IL-4 is the only cytokine that is found in higher levels in follicular lymphoma than in reactive follicular hyperplastic lymph nodes and the IL-4 receptor gene is upregulated in follicular lymphoma cells. It has experimentally been shown that T_{FH} support and protect the B-cell derived follicular lymphoma cells, not only through IL-4 but also through CD40L which interacts with CD40 on the malignant B cells and protects them from chemotherapy-induced apoptosis. The T_{FH}'s signalling lymphocyte activation molecule-associated protein (SAP) is crucial for sustaining the follicle centre: without SAP, there are no stable interactions between T_{FH} and B cells. Paper II showed worse outcome for patients with more CD4+ cells, especially when the CD4+ cells were follicularly located (i.e. T_{FH}) and even more so when follicular PD-1+ T cells and Tregs were added to the multivariate model. This adjustment for the immunosuppressive sub-subsets of CD4+ T-cells crystallised the adverse B-cell-promoting effect of the also CD4+ T_{FH}. The paramount importance of the balance between IL-4–T_{H2}–T_{FH} on one
hand and IFN-γ–CTLs–T_{H1} on the other are shown in paper II with the CD4/CD8 ratios and follicular/interfollicular CD4 ratios: higher values of both ratios correlated strongly with poor outcome. In fact, these ratios were the best predictors for outcome (Figure 4-1:A), but because of their compound nature they were not included in multivariate analysis.

Tregs, both follicular and interfollicular, were associated with good prognosis, although they were only independent in the follicular compartment. Tregs negatively regulate T_{FH} and T_{FH}-dependent B-cell survival and directly suppress B cells. Our independent results confirmed the importance of PD-1+ cells. PD-1 and its ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), constitute an important inhibitory pathway in T-cell immunity, promoting T-cell tolerance. PD-1 is detected in some T-cell lymphomas of T_{FH} origin, and is mostly expressed in CD4+CD25- (but FOXP3 in CD4+CD25+) cells. The intrafollicularly concentrated PD-1+ cells in follicular lymphoma could represent a subset of T_{FH}, different from the majority of B-cell stimulatory T_{FH}. PD-L1 is expressed in several cell types, including Tregs, but not in follicular lymphoma cells. PD-L2 is only expressed in dendritic cells, macrophages and mast cells. PD-1–PD-L-interaction leads to negative regulation of the PD-1+ cell, but the PD-1+ cell can also affect its surroundings via reverse signalling through PD-L2 and PD-L1. PD-1–PD-L-ligation leads to reverse signalling into dendritic cells, inhibiting the dendritic cells’ induction of the CD4+ helper T-cell response. In summary, PD-1+ and FOXP3+ cells are CD4+ subsets whose prognostic importance in follicular lymphoma is plausibly due to their inhibition of other immune cells that otherwise assist follicular lymphoma cells. In paper II, CD57+ cells had no association with outcome, but with higher FLIPI, similar to a previous report. CD57 is expressed in a subset of T_{FH}, but the function of the CD57 molecule and the T cells expressing this NK-cell marker is uncertain. CD4+CD57+ cells have inconclusively been called both an anergic (incapable of producing B-cell helper cytokines) and energic (highly expressing inducible costimulator [ICOS]) T_{FH} subset. PD-1 expression is stronger in CD57+ cells than in other T_{FH}. T cells coexpressing PD-1 and CD57 have been observed in follicular lymphoma. PD-1+ and CD57+ cells seem to be two distinct but sometimes overlapping subsets (Figure 4-1:B). Finally, interfollicular CD68+ macrophages correlated with inferior outcome. Classically activated (M1) macrophages, following exposure to IFN-γ, have tumoricidal activity. In response to IL-4, macrophages instead undergo alternative (M2) activation. In general, M2
Macrophages are oriented towards immunoregulation and tumour promotion. IL-4-producing Th2–TFH and also B cells drive the polarisation of macrophages to an M2-biased phenotype. Tumour-associated macrophages usually have an M2 phenotype and they induce angiogenesis and tolerance in CTLs.

To sum up the unifying microenvironment model based on the results in paper II, there is an interaction between TFH and follicular lymphoma cells, mediated through IL-4. Higher numbers of TFH entail more IL-4 and thus a propagation of the malignant B cells, and also a skewing of macrophages to an M2 phenotype which together with IL-4 inhibit CTLs and Th1. Through IFN-γ, CTLs and Th1 compete against the follicular lymphoma cells, the TFH and the macrophages. This competition is also influenced by other cytokines such as IL-10 and IL-12. The importance of the balance between Th2–TFH and Th2–CTLs is demonstrated with the CD4/CD8 and follicular/inter-follicular CD4 ratios. Two scarce immunosuppressive subsets, Tregs and PD-1+ T cells inhibit the TFH and maybe also directly the lymphoma cells.

The microenvironmental cells that probably are the most essential for follicular lymphoma survival are not immunologic but stromal. The FDCs and also other stromal cells support both follicular lymphoma cells and their normal counterparts, germinal centre B-cells. IFN-γ overrules the growth-promoting effect of stromal cells on follicular lymphoma cells. The fundamental biology and behaviour of FDCs has not yet been defined. However, the extent of FDC networks does not correlate with outcome, probably because an FDC network is a conditio sine qua non for follicular lymphoma. Follicular lymphoma cells that have migrated to the bone marrow either recruit FDCs or cause local stromal cells to differentiate into FDCs, because FDCs are sometimes found in follicular lymphoma infiltrates in the bone marrow, a compartment normally alien to FDCs. Follicular lymphoma cells can in vitro also be maintained by other stromal cells that have been caused by the lymphoma to differentiate from mesenchymal stem cells into fibroblastic reticular cells which mediate immune cell migration, adhesion, and reciprocal interactions. CXCR5+ TFH and follicular lymphoma cells are chemotactically attracted to the follicle centre with C-X-C motif chemokine 13 (CXCL13). CXCL13 is secreted by FDCs, but also by TFH and follicular lymphoma cells. CXCL13 will cause the accumulation of these cell types around the FDCs and the establishment of a pernicious follicular ménage à trois (Figure 5-1).
CTLs, PD-1+ T cells and Tregs are important in halting disease progress, in the years before whatever treatment will be needed. However, some caution is needed when we consider the microenvironment. The studies in Table 5-1 are correlational, not causal, and do not confirm each other much. The subsets for which directly follicular lymphoma-affecting effects have been demonstrated are T_{H2}–T_{FH} (through IL-4\textsuperscript{109-112} and CD40L\textsuperscript{116-119}) and CTLs (through IFN-\(\gamma\)\textsuperscript{150}) and stromal cells/FDCs (unknown mechanisms).\textsuperscript{101-108} The results for Tregs and (recently also PD-1+ cells)\textsuperscript{174} are more conflicting. It is possible that the follicular PD-1+ cells just represent end-differentiated and exhausted T_{FH},\textsuperscript{175} and that the PD-1+ cells do not exert much influence on their microenvironment, but that they correlate with prognosis only because their numbers represent the fraction of T_{FH} that have become anergic. Finally, different treatments may have different effects on different immune cells. de Jong et al. noted non-significant trends of changing prognostic properties depending on the type of treatment, with macrophages having a trend for longer progression-free survival in patients treated with CVP but a trend for shorter progression-free survival in those treated with fludarabine.\textsuperscript{146} Thus, it was suggested that the contradicting findings for prognostic markers were to be explained by differences in therapy between the studies.

**Figure 5-1: A unifying microenvironment model.**

The balance between IL-4 and IFN-\(\gamma\) decides the differentiation of CD4 to either T_{H1} or T_{H2}–T_{FH}. IL-4 is produced by T_{H2} and T_{FH}, but IFN-\(\gamma\) by CTLs and T_{H1}. The left hand path: IL-4 and T_{FH} dominate a follicular lymphoma node, pushing macrophages to the M2 phenotype and inhibiting the CTLs and T_{H1}. Inside the follicle centre FDCs, T_{FH} and follicular lymphoma (FL) cells accumulate around the FDCs via the chemotactic CXCL13–CXCR5 system. The malignant B cells thrive in the vicinity of the FDCs (unknown mechanisms) and T_{FH} (CD40–CD40L and IL-4): a ménage à trois. PD-1+ T cells\textsuperscript{148} and (some) Tregs also express CXCR5 and are recruited to the follicle centre and inhibit the T_{FH}, macrophages and FL cells. The right hand path: a situation where IFN-\(\gamma\) has supremacy, resulting in more T_{H1} and M1 macrophages and fewer T_{FH} and M2 macrophages, and ultimately less B-cell help. IFN-\(\gamma\) also inhibits the stromal FDCs' nursing of the FL cells and prevents the FL cells from migrating to new sites.
5.2 THE MICROENVIRONMENT AFTER RITUXIMAB (PAPER III)

Rituximab represents the most important advance in the treatment of follicular lymphoma in the past 30 years. Few patients in the reports in Table 5-1 received rituximab, which is now part of all standard first-line regimens. Table 5-2 shows the microenvironment studies performed on patients receiving rituximab as first-line therapy.

Table 5-2: Studies correlating outcome with cellular subsets in the immune microenvironment in upfront rituximab-treated patients.

<table>
<thead>
<tr>
<th>First author of report(s)</th>
<th>Therapy</th>
<th>Macrophages</th>
<th>T cells (CD3+)</th>
<th>CD4+ Helper T</th>
<th>CD8+ CTLs</th>
<th>FOXP3+ Tregs</th>
<th>Mast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taskinen175-178</td>
<td>CHOP</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>R-CHOP</td>
<td>G</td>
<td>N</td>
<td>STAT5a: G</td>
<td>-</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Canioni179</td>
<td>CHOP</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R-CHOP</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sweetenham180</td>
<td>Pro-MACE-MOPP</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R-CHOP/T-CHOP</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Wahlin (Paper III)</td>
<td>Rituximab</td>
<td>-</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>IFN-α-Rituximab</td>
<td>-</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

ProMACE-MOPP denotes prednisone, methotrexate, doxorubicin, cyclophosphamide, and etoposide/mechlorethamine, vincristine, procarbazine, and prednisone and T-CHOP denotes CHOP followed by consolidation with tositumomab/iodine I-131 tositumomab (Bexxar®). Association with outcome is denoted as P (poor), G (good) and N (none or not significant). Underlined findings were significant in multivariate analysis.

The addition of rituximab to CHOP thus seems to have reversed or at least cancelled the negative impact of tumour-associated macrophages. Conversely, mast cells were demonstrated to be independently associated with inferior progression-free survival in patients treated with R-CHOP but not in those treated without rituximab.147,177 A high mast cell content has been correlated with increased angiogenesis.181,182

In the study presented in paper III, we investigated the prognostic properties of T cells in lymph nodes and blood in patients given rituximab and IFN-α in the NLG trials. Higher levels of both CD4+ T cells and CD8+ CTLs in the pre-treatment lymph nodes correlated with fast and good treatment responses (Table 4-4). The CTLs were independently associated with good responses in patients who only received rituximab, but had no bearing on responses in those who also were given IFN-α priming (Figure 4-2). The favourable effect of CD4+ T cells was seen in all patients, and not affected by IFN-α. High CD4/CD8 ratios were associated with good responses only in patients who received IFN-α.

These results suggest that subsets in the microenvironment change their prognostic properties when anti-CD20-antibodies enter the malignant lymph node. Rituximab’s mechanisms of action include complement-dependent cytotoxicity, direct apoptosis, and antibody-dependent cellular cytotoxicity (ADCC).132,183 ADCC is dependent on
antibody–Fc receptor interactions. CD8+ CTLs could enhance ADCC through their production of IFN-γ.\textsuperscript{184} Rituximab also modulates the CD4+ T-cell population to a phenotype with less CD40L expression\textsuperscript{185} and towards an increased ratio of TH1 over TH2–TFH (measured as the ratio between IFN-γ+ and IL-4+ on CD4+ cells).\textsuperscript{186} Macrophages, having Fc receptors, participate in ADCC and would act favourably with rituximab to eradicate the malignant cells.\textsuperscript{132,187} It also possible that rituximab indirectly, through its modulation of the CD4+ T cells, drives the differentiation of macrophages away from the tumour-promoting M2 and towards the tumoricidal M1 phenotype. Tregs and PD-1+ cells are immunosuppressive, mostly by inhibiting other subsets such as TFH, dendritic cells and macrophages.\textsuperscript{157,160} According to the unifying model, these immunosuppressive effects are favourable at least in a wait-and-watch phase. However, the mechanisms of rituximab demand the cooperation of an active immune system. It can be speculated that when patients receive rituximab, PD-1+ cells (which also may be CTLs\textsuperscript{188}) and Tregs (that also may inhibit CTLs\textsuperscript{188,189}) will, if not modulated, have less beneficial effects. Indeed, Tregs do not appear retain a favourable impact in patients treated with R-CHOP.\textsuperscript{178,180}

The maximal clinical response to rituximab may take several months, suggesting that these short-term mechanisms are not the only ones involved. A vaccinal effect of rituximab has been proposed: rituximab-induced lysis of lymphoma cells promotes uptake and cross-presentation of lymphoma-cell derived peptides by dendritic cells, inducing their maturation, and allowing the generation of specific anti-lymphoma T cells.\textsuperscript{132} The vaccinal hypothesis was recently supported by the finding of follicular lymphoma idio-type-specific T cells subsequent to rituximab treatment.\textsuperscript{190} Dendritic cells would be pivotal in a vaccinal response, because not only helper T but also CTL activation requires dendritic cells.\textsuperscript{191} CD4+ helper T cells are needed for the generation of effective anti-lymphoma CD8+ memory cells, through dendritic cell-activation and cytokines such as IL-2.\textsuperscript{192} In summary, both CD4+ helper T cells and CD8+ CTLs would be instrumental in a vaccinal anti-lymphoma immune-response, but they would not be the only immune cells involved.

Paper III shows that IFN-α improved late treatment responses, at EV-2, after eight doses of rituximab. IFN-α has countless effects, including the enhancement of the expression of MHC class I proteins and the proliferation and promotion of CD8+ CTLs.\textsuperscript{193} An IFN-α-driven expansion of CTLs could explain why the addition of IFN-α
had maximal therapeutic effects in patients with few CD8+ CTLs. The pre-treatment
CTL levels were independently predictive for better outcome in patients given only
rituximab but irrelevant in patients also receiving IFN-α. IFN-α is known to exert an
antiproliferative effect on CD4+ cells, at least in vitro,\textsuperscript{194} which could mean that higher
pre-treatment CD4+ levels are needed when IFN-α is given. Taken together, this would
explain why high CD4/CD8 ratios correlate with better outcome in IFN-α-rituximab-
treated patients. IFN-α has been suggested for use as an adjuvant to obtain anti-tumour
vaccinal effects.\textsuperscript{132,193} The results in paper III are in line with a slow, vaccine-like effect
of IFN-α, only apparent many months after the initiation of therapy. Other important
effects of IFN-α are its suppression of IL-4 production in CD4+ cells and its anti-
angiogenic action in tumours,\textsuperscript{193,195} and IFN-α seems to abrogate the negative effect of
angiogenesis in follicular lymphoma,\textsuperscript{196} which otherwise has been correlated with poor
prognosis,\textsuperscript{197-199} also in patients treated with R-CHOP.\textsuperscript{181}

5.3 BEYOND THE MICROENVIRONMENT: SYSTEMIC T CELLS AND
RITUXIMAB (PAPER III)

Paper III presented an association between a fast response to rituximab and tumour T-
cells. That implies that cells already present in the microenvironment are the first to
contribute to rituximab-induced lymphoma-killing. However, tumour-associated T-
cells seemed less predictive for responses at later timepoints and showed no bearing on
survival. Because no study has yet shown any microenvironmental factor to be pro-
gnostic for overall survival in patients given rituximab, we suggest that rituximab could
be a “microenvironment equaliser”. Rituximab and its inflammatory mechanisms may
recruit systemic immune cells and induce changes of local immune cells, rendering the
pre-treatment immune cell patterns irrelevant for further prognostication. The microen-
vironment may be irreversibly altered after rituximab. In contrast, paper III showed that
systemic T-cell levels, as measured in the blood, correlated with good but slower
responses and also with better overall survival (CD3+ and CD4+ T cells) and time to
next treatment (CD8+ CTLs; \textbf{Figure 4-3}). The blood T-cells also seemed to have a con-
nection with the lymphoma even before therapy commenced, because they were
strongly associated with tumour-specific factors such as lactate dehydrogenase, number
of involved nodal areas and haemoglobin levels. The blood T-cells must better reflect
the patient’s immune system than the T-cells in the disturbed tumour microenviron-
ment. It should be remembered that the lymph node and blood measurements represent
different kinds of T cells (especially with respect to CD4+ T cells): before any therapy,
the CXCR5+ TFH in contact with the follicular lymphoma cells have been recruited to
the diseased follicle centres by CXCL13, while the blood CD4+ T cells are unaffected
by the disease. After anti-CD20 therapy, systemic T cells will be attracted to the local
environment by signals arising from the anti-lymphoma killing processes. The blood
may be the pool from which lymphoma idiotypic-specific T cells are mustered after
rituximab. A summary of the events after rituximab and IFN-α have entered the system
is partly guesswork. However, it seems that rituximab not only eradicates the B-cells
but also skews the IL-4/IFN-γ balance (Figure 5-1) so that the differentiation of naïve
CD4+ T cells and macrophages turn from Th2–TFH and M2 and towards Th1 and M1.
This ought to be both a local and a systemic effect. The modulation towards less IL-4-
producing CD4+ phenotypes is enhanced with IFN-α, which also abrogates the
negative effects of few CTLs and rich angiogenesis. Rituximab needs immune cells for
full anti-lymphoma effect. Macrophages participate in ADCC. Local CD4+ T cells and
CD8+ CTLs improve the response to rituximab, maybe through production of IFN-γ.
After some months the vaccinal effect sets in, helped by the short courses of IFN-α
priming. High pre-treatment blood T-cell levels seem particularly important for this late
effect. With therapies utilising the immune system, host-specific immune factors may
be more important than the cells in the disturbed local microenvironment, which will be
wiped out after therapy.

5.4 GRADE 3B IS A DISTINCT AGGRESSIVE BUT CURABLE ENTITY
(PAPER IV)

In paper IV, we presented a pathology review of 828 patients with follicular lymphoma.
Thirty-two patients had follicular lymphoma grade 3B and, of these, 23 had purely
follicular disease. We showed that grade 3B is a disease with much higher initial mortal-
ity than grade 1–3A (Figure 4-4). The median overall survival was 13.0 years for grade
1–3A patients (12.2 years for grade 3A patients) and 4.4 years for grade 3B patients.
Patients with grade 3B did not follow an indolent course and some of them seem to be
cured, because there were no relapses and no deaths beyond five years of follow-up.
Grade 3B patients who received first-line anthracycline-containing therapy had much
better overall survival at five years compared with those who did not (56% vs 14%).
Grade 3B was independent in multivariate analysis (Table 4-6). All reports investigating
clinical differences between purely follicular grade 3A and 3B are shown in Table 5-3.
Table 5-3: Investigations of clinical differences between grade 3A and 3B.*

<table>
<thead>
<tr>
<th>First author of report</th>
<th>No. of grade 3A patients</th>
<th>No. of grade 3B patients</th>
<th>Fraction of grade 3B of grade 3</th>
<th>Median follow-up time (years)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chau32</td>
<td>44</td>
<td>11</td>
<td>20%</td>
<td>7</td>
<td>Survival curves diverge. Grade 3B never relapses after anthracyclines.</td>
</tr>
<tr>
<td>Hans33</td>
<td>76</td>
<td>25</td>
<td>25%</td>
<td>6</td>
<td>Survival curves diverge; short follow-up.</td>
</tr>
<tr>
<td>Hsi34</td>
<td>35</td>
<td>10</td>
<td>22%</td>
<td>2</td>
<td>Survival curves diverge; very short follow-up.</td>
</tr>
<tr>
<td>Shustik35</td>
<td>139</td>
<td>22</td>
<td>14%</td>
<td>4</td>
<td>Survival curves do not diverge; short follow-up.</td>
</tr>
<tr>
<td>Wahlin (Paper IV)</td>
<td>94</td>
<td>23</td>
<td>20%</td>
<td>9</td>
<td>Survival curves diverge significantly. Improved survival in 3B after anthracyclines.</td>
</tr>
</tbody>
</table>

* Patients with purely follicular grade 3 are included (i.e. patients with grade 3 and concomitant DLBCL are excluded).

Although paper IV is the first report to show statistically significant different outcomes in grade 3A and 3B, trends of different mortality rates between grade 3A and 3B could be seen in three32-34 of the four previous studies. These studies were limited by few grade 3B patients or short follow-up times. Chau showed that none of the four grade 3B patients who had received first-line anthracycline-based chemotherapy experienced disease relapse or progression, but this was not statistically significant in such a small population.32 The reports of Hans33 and Hsi34 showed apparent inferior survival in grade 3B patients. Median overall survival was reported by Hans to be in grade 3A about 10.0 years and in grade 3B about 6.5 years. The corresponding numbers reported by Hsi were 3.8 and 1.8 years. These studies were limited by short follow-up. The survival curves of the two subtypes of grade 3 did not diverge in the study by Shustik.35 However, this study was also hampered by short follow-up time, and maybe by under-representation of grade 3B cases (only 14% of all grade 3 were 3B). The WHO classification states that in unselected series of purely follicular grade 3, the expected fraction of grade 3B cases is 20%–25%.13 Thus, our results broadly agree with most of the previous results. Our study is the first to show a plateau in the survival curve of grade 3B patients, and we believe that is only possible to do in patients followed for long times. The median follow-up for the grade 3B patients in our study was 9.4 years (range, 6.7–16.0). Our minimal follow-up time of 6.7 years is larger than the longest previously published median follow-up time (Chau, 6.6 years). If the follow-up had been shorter, the survival curves of paper IV would probably have looked more like those presented by Hans and Hsi.

WHO defines grade 3B by “solid sheets of centroblasts” but also says that “grade 3B follicles are composed entirely of large blastic cells (centroblasts or immunoblasts).”13 We examined the grade 3 cases using both the solid-sheet and the entire-follicle
criteria. All grade 3B patients appeared to suffer from aggressive disease, both those whose follicles were entirely populated with centroblasts \((n=13)\) and those who were only defined by the presence of solid sheets of centroblasts \((n=10)\). In patients who were not given any anthracyclines upfront, either of the subcategories of grade 3B correlated significantly with worse overall survival compared with grade 1–3A. Finally, there was more positivity for Ki67 and p53 and less for BCL-2 in grade 3B biopsies.

In contrast to grade 1–3A (but similarly to DLBCL), grade 3B affects fewer women than men. An analysis of all previously published gender distributions\(^{33-35}\) together with that in paper IV shows that women represent 55% of grade 3A patients but 43% of grade 3B patients (compared with 58% of follicular lymphoma patients and 45% of DLBCL patients\(^{53}\)). The low frequency of bone-marrow involvement in grade 3B (17%) is also different from the situation in grade 1–3A (44%) but similar to reports of that in DLBCL (17\%\(^{53}\)). Analogously, there is also less stage III–IV disease in grade 3B, as already shown in entities of older nomenclatures corresponding to grade 3B.\(^{30,39}\)

There are underlying genetic differences between grades 1–3A and 3B. Grade 3B has much lower frequencies of \(t(14;18)\) than grade 3A.\(^{48,49}\) Chromosomal breaks at 3q27, rearranging BCL-6, are more common in grade 3B.\(^{48}\) It has been suggested that CD10+ grade 3B cases with \(t(14;18)\) are more related to grades 1–3A, while those with 3q27 breaks are a separate entity.\(^{200}\) However, 3q27 breaks have predominantly been found in grade 3B cases with concomitant DLBCL, whereas pure grade 3B cases typically lack either abnormality.\(^{201}\) Gene expression in grade 3B is distinct from that in both grades 1–3A and DLBCL.\(^{50}\) Taken together, grade 3B has not only a divergent clinical course compared with grades 1–3A, but is also different with respect to epidemiology (more males), clinical characteristics (less bone-marrow involvement), and genetic features. We propose replacing the term “follicular lymphoma grade 3B” with “follicular large B-cell lymphoma”. Follicular large B-cell lymphoma is not a subtype of follicular lymphoma but a separate disease entity. It is aggressive but curable with anthracyclines, and patients with this disease should be treated as those with DLBCL. This conclusion is in line with many older reports which showed the highest grade of follicular lymphoma, then often called follicular large cell lymphoma, to be clinically similar to DLBCL and curable with intensive therapy.\(^{26,32,36-42}\)
5.5 GRADE 3A IS AS INDOLENT AND INCURABLE AS GRADE 1–2
(PAPER IV)

Paper IV showed that follicular lymphoma grade 3A is an indolent disease, incurable with conventional therapy, and it may relapse after many years in remission. Patients with grade 3A did not seem to profit from first-line anthracyclines more than grade 1–2 patients did and they did not attain long-term cure, agreeing with previous studies.\textsuperscript{32,35} Some patients with grade 3A survive untreated for decades. Follicular lymphoma grade 3A is as indolent and incurable as grades 1 and 2. There is no rationale for treating grade 3A more aggressively than grade 1–2. Furthermore, increasing Ki67-positivity, which increased with higher grades, did not predict survival, in agreement with most previous studies.\textsuperscript{24,31,45-47}

5.6 RITUXIMAB DIFFERENTIALLY AFFECTS PATIENTS WITH DIFFERENT GRADES (PAPER IV)

In patients given upfront rituximab-containing therapy, higher grades of 1, 2, and 3A were associated with better overall survival and time to treatment-failure, independently of other factors (Figure 4-5; Table 4-7). The results were very alike in the large subset of patients receiving rituximab without chemotherapy in the randomised NLG trials. Even in the relatively few patients treated upfront with rituximab and chemotherapy, higher grades were significantly associated with longer time to treatment-failure. Furthermore, similar trends were seen in both cohorts, which were graded by different pathologists. This gives some validation to our findings. Positivity for the proliferation marker Ki67 also showed a favourable trend in rituximab-treated patients. Our results are in contrast with what could be expected based on publications from the pre-rituximab era. But, interestingly, in patients treated with single alkylators upfront, higher grades correlated with inferior overall survival (patients with grade 1 fared better than patients with grades 2 and 3). Thus, with this (older) non-intensive chemotherapeutic regimen, the lowest grade correlated with better outcome, while with rituximab without chemotherapy, also a comparably non-intensive regimen, increasing grades correlated incrementally with better outcome. The dividing thresholds of five and 15 centroblasts/hpf were originally arbitrarily chosen,\textsuperscript{23} and neither seems to be a particularly important breaking point for prognosis. Rather, there seems to be gradual differences over the entire spectrum of grade 1, 2 and 3A follicular lymphoma. However, the established grading system provides a useful ordinal scale for assessing the centroblast/centrocyte ratio. Information may be lost with a dichotomous scale of grade
1–2 vs grade 3A. Thus, we argue that follicular lymphoma grade 1 and 2 should be kept separate and not merged into one entity (grade 1–2), contrary to what is approved by the current WHO classification.13

5.7 RETURN TO THE MICROENVIRONMENT? (PAPER IV)

Like the grades, tumour-associated macrophages have been associated with inferior survival in patients treated without135,141,179 but not in those treated with176,179 upfront rituximab. Macrophages are believed to act synergistically with anti-CD20-targeted therapy.176,179 Interestingly, the numbers of tumour-associated macrophages are more common in higher grades.135,140,141 Also other subsets differ: Tregs,137 PD-1+,138 and CD57+140 T cells are all less numerous in higher grades. This raises the possibility that in the rituximab setting, the causal link between higher grades and longer survival could be found in nonmalignant features of the immune microenvironment. However, we argued above that rituximab will cause considerable immigration of systemic (and modulation of) immune cells, and, after exposition to rituximab, it could be problematic to attribute long-term prognostic strength to pre-treatment microenvironmental immune cell levels. There could also be properties in the tumour cells themselves that lend them more or less susceptible to different types of treatment. Some of these properties may covariate with or depend on the centroblast/centrocyte balance or the number of proliferating tumour cells.
6 CONCLUSION

6.1 THE BALANCE BETWEEN IMMUNE CELLS IN THE MICROENVIRONMENT PREDICTS OUTCOME

Competition between different immune cells in the microenvironment affects the outcome in follicular lymphoma. The CD8+ CTLs, FOXP3+ Tregs and PD-1+ T cells inhibit disease progression by restraining the CD4+ T_{FH} and CD68+ macrophages that promote the follicular lymphoma. Clinical and laboratory evidence suggests a central IL-4-dependent axis between T_{FH} and follicular lymphoma cells. IL-4 also modulates macrophages into the tumour-promoting M2 phenotype, and together with these inhibits the CTLs. CTLs (and the interfollicular CD4+ T_{H1}) produce IFN-γ which counteracts IL-4 and the IL-4-driven T_{FH}–lymphoma crosstalk and also overrules the FDCs’ nursing of follicular lymphoma cells and pushes macrophages towards the tumoricidal M1 phenotype. The balance between IL-4–T_{H2}–T_{FH} and IFN-γ–CTLs–T_{H1} determines disease progression. This is demonstrated with the poor outcome associated with higher ratios of CD4/CD8 and of follicular/interfollicular CD4. Tregs and PD+1 T cells could be favourable because inside the follicles they inhibit the T_{FH} and maybe also the tumour cells.

6.2 RITUXIMAB AND IFN-Α ALTER THE PROGNOSTIC PROPERTIES OF THE MICROENVIRONMENT

Rituximab treatment cancels the negative impact of CD4+ T_{FH}, likely because they are modulated to a less B-helping phenotype, thus nullifying the importance of the pre-rituximab balance between IL-4–CD4 and IFN-γ–CD8. Furthermore, CD4+ helper T cells and CD8+ CTLs would participate in the postulated rituximab-induced vaccine-like immune response. The addition of IFN-α to rituximab enhances late responses, and makes rituximab less dependent on pre-treatment CTL levels. As of yet, in patients who received rituximab in first line, no study has demonstrated an impact from micro-environmental factors on overall survival. Is rituximab a microenvironment equaliser?
6.3 **BLOOD T CELLS ARE PROGNOSTIC IN PATIENTS TREATED WITH RITUXIMAB AND IFN-α**

CD4+ and CD8+ T cells in blood, as well as their total sum, measured as CD3+ T cells, correlate with late, good responses after rituximab. IFN-α abrogates the importance of CD8+ CTLs in blood (as well as in lymph nodes). Blood CD3+ and CD4+ T cells predict better overall survival. Blood CD8+ CTLs predict time to next treatment (CD3+ and CD4+ cells show similar trends). Higher blood T-cell levels are also associated with low-risk FLIPI scores.

6.4 **GRADE 3B IS AN AGGRESSIVE BUT CURABLE ENTITY:**

**FOLLICULAR LARGE B-CELL LYMPHOMA**

Grade 3B is a biologically and clinically distinct lymphoma entity. It is aggressive but curable with anthracyclines and autologous stem cell transplantation. It should be recognised as a separate entity; we propose the name follicular large B-cell lymphoma. Follicular large B-cell lymphoma is to be treated as DLBCL.

6.5 **FOLLICULAR LYMPHOMA GRADE 3A IS AS INDOLENT AND INCURABLE AS GRADE 1 AND 2**

Follicular lymphoma grades 1, 2 and 3A represent a single indolent and with conventional therapy incurable disease but also increments in an established and useful ordinal scale of increasing centroblast/centrocyte ratio. The centroblasts in grade 1, 2 and 3A are not “transformed cells”.

6.6 **INCREASING GRADES OF 1, 2 AND 3A PREDICT BETTER OUTCOME IN THE RITUXIMAB ERA**

Increasing centroblast/centrocyte ratio, as measured with the current 2008 WHO grading system, is an independent predictor for better overall survival and time to treatment-failure after rituximab-containing therapy, even without chemotherapy.
7 FUTURE PERSPECTIVES

7.1 DELAY THE START OF THERAPY: THE “WAIT-AND-BRAKE” PHASE

Before the onset of symptoms necessitating therapy, patients with advanced disease are managed in a wait-and-watch phase. IFN-α has been successfully tried for delaying the onset of symptoms, but the side effects with long-time IFN-α have hindered its implementation. The follicular lymphoma cells’ ability to create their environment decides the progress of the disease. Better knowledge of the mechanisms with which the follicular lymphoma cells interact with FDCs and T<sub>FH</sub> could provide more specific targets for disease-slowing medications. Instead of wait-and-watch, we would have a “wait-and-brake” phase, maybe delaying symptomatic disease and the need for aggressive treatment for years.

7.2 THE IMMUNE SYSTEM IN FOLLICULAR LYMPHOMA IN RELATION TO THERAPY

It is likely that the local microenvironment’s interactions with the malignant cells decide outcome before therapy is started. Chemotherapy destroys many immune cells. Biologic therapy, such as rituximab, both modulates and recruits immune cells to the tumours. In the long run, therapeutic effects may be decided by host-specific factors, such as the variation in Fcγ receptors and the T cell repertoire.

7.3 BOOST THE EFFECT OF ANTI-CD20 THERAPY

Rituximab or some other specific anti-B cell monoclonal antibody will remain the mainstay of treatment for patients with follicular lymphoma in the foreseeable future. Rituximab without chemotherapy yields long-term remissions in some, but not all, patients. We believe our findings suggest a future of personalised therapy based on biological characteristics. Some patients may be identified who do not need the addition of chemotherapy to anti-CD20 antibodies. Other patients may have their immune systems reinforced with adjuvants that abrogate host-specific and tumour-induced weaknesses to boost the effectiveness of antibodies. Not only IFN-α but also lenalidomide and granulocyte-macrophage colony-stimulating factor (GM-CSF) seem to reinforce and restore immune cells and potentiate rituximab.
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