Clinical Significance of T-Regulatory Cells in B-Cell Non-Hodgkin's Lymphoma

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Regulatory T cells (Tregs) play a central role in protecting the host from autoimmune diseases. However, Tregs also pose a major problem to anti-tumor immunity. The expansion and accumulation of these immunosuppressive cells correlate with advanced tumor growth and predict poor disease prognosis. The study aims at evaluation of the clinical role of regulatory T cells in B-cell Non-Hodgkin's lymphoma. The study was carried out on 45 de novo patients with B-NHL, they included 26 males and 19 females and 20 apparently healthy age-matched as control. Diagnosis of lymphoma was done by lymph node biopsy, 15 patients had diffuse large B cell lymphoma (DLBCL), 9 follicular lymphoma (FL), 8 small cell lymphocytic lymphoma (SCLL), 7 marginal zone lymphoma (MZL) and 6 had mantle cell lymphoma (MCL). Tregs (CD4+CD25+FoxP3) were analyzed by Flowcytometry. The mean percent was 10.9±1.6% in diffuse large B cell lymphoma, 12.4±1.4% in follicular lymphoma, 13.7±2.4% in small cell lymphocytic lymphoma, 11.9±1.9% in marginal zone lymphoma and 13.5±1.35% in mantle cell lymphoma as compared to 5.9±0.96% in controls with a significant increase in the patients in relation to control group. Tregs was significantly increased in advanced stages of the disease, in bone marrow infiltration and in patients with increased LDH level. In conclusion, Tregs may play a role in modifying immune responses in patients with lymphomas and may be useful in immunotherapy and new anti-lymphoma strategies involving depletion of Tregs.

B cell non-Hodgkin's lymphoma (B-NHL) is a heterogeneous group of lymphoid malignancies, many of which remain incurable. Increasing evidence suggests that the tumor microenvironment plays an important role in determining not only the severity of disease, but also response to therapy, (de Jong, 2005).

Ansell et al., (2001), demonstrated that higher numbers of activated CD4+ cells among the tumor-infiltrating lymphocytes of diffuse large B-cell NHL were associated with a better prognosis.

Regulatory T cells (Treg) are a group of immune suppressive cells that have been intensively studied in recent years. Treg is closely correlated with autoimmune diseases and tumors. Most previous studies have labelled CD4 and CD25 on Treg, but CD25 is also expressed on the surface of functional T cells. The accuracy and robustness of Treg detection by CD4 and CD25 labelling studies suggested that CD127 expression on cell surface was negatively related to the forkhead/winged helix transcription factor p3 (FoxP3), while FoxP3 has been proven the most reliable and specific marker of Treg. Treg shows low level expression of CD127, while auto-activated T cells have high level expression of CD127, (Hui, et al., 2009).

Precise understanding of the immunosuppressive mechanism of Treg cells remains elusive, although there is increasing evidence that Tregs manifest their function through a myriad of mechanisms that include the secretion of immunosuppressive soluble factors such as IL-9, IL-10 and TGFβ, cell contact mediated regulation via the high affinity TCR and other costimulatory molecules such as CTLA-4, GITR, and cytolytic activity. Understanding the mechanisms by which Treg cells exert their influence is an area of intense research with broad implications for the development of therapeutic strategies for many disease processes including cancer, diabetes, and
Immune mediated diseases, (Yang, et al., 2009).

There has been accumulating evidence that CD4 CD25 FoxP3 expressing regulatory T cells (Treg) are highly concentrated in tumors, thereby fostering an immune-privileged microenvironment. Some studies have shown that T-cell receptor (TCR) stimulation can convert conventional T cells into Treg. Follicular lymphoma (FL) B cells can enhance this Treg conversion, (Weiyun et al., 2009).

The aim of the work is the evaluation of the clinical role of regulatory T cells in B cell NHL.

Subjects and Methods

This study was carried out in Zagazig University Hospitals, Clinical Pathology and Internal medicine Departments. It included 45 de novo patients with B-cell NHL (age range: 15 to 72 years, mean 54.18 ± SD 15.42 years), 26 males, 19 females and 20 apparently healthy age-matched control group (age range: 17 to 70 years, mean 52.17 ± SD 14.22 years). According to the stages of the disease, 17 patients had stageI, 13 patients in stage II while 8 patients in stage III and 7 patients in stage IV (Ann Arbor staging system was used for those aged >18 years, and St Jude staging system for those aged ≤ 18). Diagnosis of lymphoma was done by lymph node biopsy and histopathological examination which showed, 15 patients were diagnosed as diffuse large B cell lymphoma, 9 had follicular lymphoma, 8 had small cell lymphocytic lymphoma, 7 had marginal zone lymphoma and 6 had mantle cell lymphoma.

After informed consent was obtained, all the patients were subjected to the following: Full history taking, careful clinical examination for lymphadenopathy with histopathological examination for removed lymph nodes, x-ray and CT examination before any antilymphoma treatment was given.

Laboratory investigations included: liver function, kidney function, complete blood count, serum LDH, Leishman stained peripheral blood smears and bone marrow aspirates, were examined for presence of abnormal cells (lymphoma Cells) and immunophenotyping was performed if needed.

Lymphocyte Separation

Heparinized blood sample was taken from all participants for Treg detection. PBMCs were purified by Ficoll-Hypaque gradients (Seromed-Biochrom, Berlin, Germany). Separated PBMCs for flowcytometric assay was washed twice with FACs washing solution; the cell pellet was suspended in FACs buffer at a concentration of 1.0 ×10⁶ /ml.

Treg Detection by Flowcytometry

Treg Markers were detected by using specific fluorochrome conjugated mouse anti-human monoclonal antibodies (mAb), peridinin chlorophyll (Per-CP) conjugated anti-CD3, Allophycocyanin (APC) conjugated anti-CD25, Fluorescein isothiocyanate (FITC) conjugated anti-CD4, and Phycoerythrin (PE) conjugated anti-FoxP3 (eBioscience San Diego CA).

- Sample preparation and staining
  Surface staining was performed for detection of CD3, CD4, CD25, by adding 20 µl of each mAb to 100 µl of separated PBMCs, followed by incubation for 30 min in the dark at 4ºC, then the tubes were washed twice with FACs washing buffer. Intracellular staining was done for detection of FoxP3 according to the manufacturer's protocol (eBioscience). Cells were first stained with surface mAb of interest (anti CD4/antiCD25) and washed twice with PBS. After permeabilization with a Fixation/Permeabilization Buffer, 20 µl anti-humans Foxp3 mAb were added, the cells were incubated for 30 min in the dark at 4ºC. Finally, 0.5 ml of phosphate buffered saline (PBS) was added to the washed cells and the samples was ready for the measurement of the CD4⁺CD25⁻ and FoxP3 using a FACScalibur flowcytometry (Becton Dickinson, San Jose, CA). To avoid nonspecific Fc receptor staining, appropriate isotype controls of mouse anti-human mAbs were used. FACs-acquisition and analysis were performed with FACs Cell Quest software (BD Bioscience). Samples were first examined for the frequency of CD3⁺CD4⁺ T-cells. The percentage of CD4⁺CD25⁻ FoxP3 T cells in the total CD4⁺ T-cells population were then determined, and the intensity of CD25 surface expression was measured using mean fluorescence intensity (MFI) as described by Sakaguchi et al., (2005).

Statistical Analysis

Data were analyzed by using software SPSS15. Data were expressed as mean±SD for quantitative variable, number and percentage for qualitative one. Analysis of variance (ANOVA or t test) was done for
comparison of means of more than two groups. P<0.05 was considered to be statistically significant.

Results
45 patients with different subtypes of B-cell NHL were included in this study as well as 20 healthy subjects as control.

The mean % of Tregs (CD4^+CD25^+ FoxP^3), in diffuse large B cell lymphoma was 10.9±1.6%, in follicular lymphoma was 12.4±1.4%, in small cell lymphocytic lymphoma 13.7±2.4%, in marginal zone lymphoma was 11.9±1.9%, while in mantle cell lymphoma, it was 13.5±1.35%, figures (1-2). In control Treg % (5.9±0.96), was statistically lower than patients (P<0.001).

Treg % was significantly increased in advanced stages of the disease, in bone marrow infiltration and in cases with increased LDH level (P<0.05). No significant difference was observed as regard age and sex, (Table 1).

ANOVA test for Treg% between different subtypes of lymphomas was showed in (Table 2).

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Figure 1. Flowcytometry analysis of CD4+CD25+ T cells. Figure 2. The purity of Foxp^+^3 Cells from gated CD25^+. 
Table 1. Characteristics and mean% of Tregs in patients with Non Hodgkin's Lymphoma and controls

<table>
<thead>
<tr>
<th>Patient's Character</th>
<th>Item</th>
<th>No</th>
<th>Treg % (mean±SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Sex</td>
<td>Male</td>
<td>26</td>
<td>11.84±1.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>19</td>
<td>12.5±2.1</td>
<td></td>
</tr>
<tr>
<td>- Bone marrow infiltration</td>
<td>Yes</td>
<td>10</td>
<td>13.7±1.4</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>35</td>
<td>11.8±1.93</td>
<td></td>
</tr>
<tr>
<td>- Stages</td>
<td>I</td>
<td>17</td>
<td>11.56±2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>13</td>
<td>11.2±1.57</td>
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</tr>
<tr>
<td></td>
<td>III</td>
<td>8</td>
<td>13.0±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>7</td>
<td>13.6±1.3</td>
<td></td>
</tr>
<tr>
<td>- LDH (mean)</td>
<td>Normal</td>
<td>15</td>
<td>10.51±1.3</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>30</td>
<td>13.0±1.86</td>
<td></td>
</tr>
<tr>
<td>- Pathological type</td>
<td>DLBCL</td>
<td>15</td>
<td>10.9±1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>9</td>
<td>12.4±1.4</td>
<td></td>
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<tr>
<td></td>
<td>SCLL</td>
<td>8</td>
<td>13.7±2.4</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>MZL</td>
<td>7</td>
<td>11.9±1.9</td>
<td></td>
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<tr>
<td></td>
<td>MCL</td>
<td>6</td>
<td>13.5±1.35</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>20</td>
<td>5.9±0.96</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

DLBCL: Diffuse large B cell lymphoma; FL: Follicular lymphoma; SCLL: Small cell lymphocytic lymphoma; MZL: Marginal zone lymphoma; MCL: Mantel cell lymphoma.
P< 0.05 is significant. NS = not significant.

Table 2. Comparison between percent Treg in different subtypes of lymphoma by ANOVA test

<table>
<thead>
<tr>
<th>Pathological type</th>
<th>DLBCL (10.9±1.6)</th>
<th>FL (12.4±1.4)</th>
<th>SCLL (13.7±2.4)</th>
<th>MZL (11.9±1.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC (13.5±1.35)</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MZ (11.9±1.9)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SCLL (13.7±2.4)</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FL (12.4±1.4)</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

DLBCL: Diffuse large B cell lymphoma; FL: Follicular lymphoma; SCLL: Small cell lymphocytic lymphoma; MZL: Marginal zone lymphoma; MCL: Mantel cell lymphoma.
P< 0.05 is significant. NS = Non significant.
Discussion

Regulatory T cells (Tregs) have an important role of suppressing effector T cells and preventing reactivity to self-antigens, Sakaguchi, (2005). These cells recognize specific tumor antigens and differentiate into cells capable of suppressing naive and CD4 Th1 anti-tumor effector cells, (Nishikawa, et al., 2005).

Before 2007, detection methods for Treg mostly involved CD4 and CD25 labelling on Treg. Since CD25 is also expressed on activated effector T cells besides on Treg, and there is no standard definition for high level expression of CD25, combined labelling of these two markers is considered an inaccurate method in detecting peripheral blood Treg level. Therefore, the results from studies that detected Treg with this labelling method are much less valuable, (Lee, et al., 2008). Now the application of FoxP3 as Treg marker makes the results more valuable.

In this study we aimed to evaluate the role of Tregs in B-cell non Hodgkin’s lymphoma (B-NHL) by detection of Tregs (CD4+CD25+ FoxP3). Tregs detection should have been done by gating on the CD25 high from total CD4.

The mean percent of Tregs in different types of lymphomas were statistically elevated in comparison to control. The results were demonstrated high numbers of cells with Tregs phenotype in the peripheral blood. The mean percentage in the five subtypes of lymphomas was 12.48 %, where in diffuse large B cell lymphoma was 10.9%, in follicular lymphoma 12.4%, in small cell lymphocytic lymphoma 13.7% but in marginal zone lymphoma, the percentage was 11.9% and 13.5% in mantle cell lymphoma. In healthy control Tregs level was 5.9 %.

It has been shown that tumor immunosurveillance is more efficient when Tregs are depleted. In human cancer effective anti-tumor responses are also affected by the presence of Tregs. These cells have been identified in increased frequency in the peripheral blood of patients with several different tumor types. Furthermore, increased density of Tregs within carcinoma biopsies is predictive of poor survival, (Curiel, et al., 2004).

The relevance of the Treg population to tumor progression in B-cell NHL is suggested in this study by close correlation between Treg numbers with both serum LDH levels and clinical staging. There is a growing evidence that the degree of Treg expansion is associated with more advanced disease. Beyer et al., (2005), found a significantly higher number of Treg cells in Binet stage C compared with Binet stage A disease in chronic lymphocytic leukemia. Nadal et al., (2007), demonstrated a higher probability of relapse in patients with chronic myeloid leukemia after allogenic transplantation with increased frequencies of CD25high Treg cells.

The relationship between Treg and lymphoma is still controversial. Both elevated and decreased Treg levels were observed; Some studies indicated positive correlation with prognosis, but opposite opinion was also suggested; Different conclusions were reached for different subtypes for peripheral blood and local tumor tissue as well, Mittal et al., (2009). However, in follicular NHL, more FoxP3 cells, assessed immunocytochemically, were associated with better survival, whereas in diffuse large B-cell lymphoma, they did not predict the outcome, (Hasselblom, et al., 2007). Tzankov et al., (2008), reported that several recent studies showed conflicting prognostic data for some haematological malignancies, specifically B-cell lymphoma, in which higher numbers of FOXP3+ cells (taken to be Tregs) were shown to correlate with improved survival. Curiously, in B-CLL it has been shown that high Treg numbers also
correlate with advanced disease stage. The differences in prognostic benefit of Tregs between haematological malignancy and carcinoma remain to be clarified. However, it has been suggested that the earlier shown suppressive effects of Tregs on B-cell function may explain the benefit of high Treg numbers in B-cell lymphoma patients. Our results showed positive correlation between Treg level and disease progression, this discrepancy of results remain unclear.

Our results seem consistent with Shi et al., (2004), who showed that the population of CD4 (+) CD25 (+)-Tregs in peripheral blood of the patients with B-NHL with or without chemotherapy was significantly higher than those in healthy donors, which may be one of the important reasons of immunosuppression in B-NHL.

Hui et al., (2009), reported that the average peripheral blood CD4+CD25^{high} CD127^{low} Treg levels were 11.25 and 8.07 % in newly diagnosed NHL patients and healthy adults. The peripheral blood level of Treg was significantly higher in the male NHL patients than in the female patients (P=0.03). There was no significant relation between peripheral blood Treg level and age, stage, LDH level, pathologic subtype. Our results showed positive correlation between Tregs numbers with both LDH level, clinical stage of the disease and pathological subtypes, but no significant relation of Treg number with age and sex.

Zhongguo et al., (2006), showed that the prevalence of CD4+CD25^{high} Treg cells was significantly higher than those in the healthy group [(4.10 +/- 1.21)% versus (2.04 +/- 1.03)%, P < 0.001]. They were concluded that the relative increase of CD4+CD25^{high} Treg cells in peripheral blood of B-NHL patients may be related to immunosuppression and tumor progression. Our results showed higher percentage of Tregs than reported previously, this may be attributed to the difference in gating strategies. In this study the Treg cells were defined by gating on the CD25 positive cells not the CD25 high positive cells.

A statistically higher Treg numbers were observed in patients than healthy controls, also in patients with increased LDH level and advanced stage of disease. Mittal et al., (2009), demonstrated that CD25^{+} FoxP3^{+} CD127^{low}CD4^{+} Treg cells were increased markedly in PBMCs versus healthy controls (P < 0.001) regardless of lymphoma subtype, and correlated with disease stage and serum lactate dehydrogenase. T-cell hypo-responsive was reversed by depleting CD25^{+} cells, or by adding anti–CTLA-4, supporting the view that Treg cells explain the systemic immunosuppression seen in NHL. A high proportion of Treg cells was also present in involved tissues versus reactive nodes (P = 0.02).

Yang et al., (2006), reported that the underlying mechanisms by which tumor cells are resistant to CTL-mediated apoptosis are not clear. Using a human model of (B-cell NHL), they showed that intratumoral Treg cells inhibit the proliferation and granule production of activated autologous infiltrating CD8^{+} T cells.

Heier et al., (2008), studied the impact of CD25^{+} Tregs in a B cell lymphoma. They showed that removal of CD25^{+} Tregs enhanced anti-tumor immunity against local growth of a B cell lymphoma and that induction or expansion of Tregs could be one mechanism by which the growing tumor evades immunosurveillance.

Modulating the action of Tregs represents one aspect in the prevention of tumor 'immune' escape, which potentially enhances immunosurveillance and the effects of other immunotherapeutic modalities. A number of agents affect Tregs in a clinical context, some of which are designed specifically to target known receptors on Tregs, whereas others, including conventional chemotherapeutic
drugs, have modulatory effects on Tregs although their exact mode of action is often unknown. Some commonly used chemotherapeutic agents, such as methotrexate and cyclophosphamide, can have immunostimulatory and anti-angiogenic effects at lower doses without the toxic effects associated with higher doses. This new concept of treatment is on the basis of the more frequent or 'metronomic' administration of a dose substantially lower than the maximum tolerated dose. Cyclophosphamide depletes CD4+CD25+ Tregs in mice injected with tolerogenic syngeneic tumour cells, (Ghiringhelli, et al., 2004).

Depletion of Tregs leads to a decrease in tumour cell growth and eventual rejection of established tumours when used in combination with an immunotherapeutic agent (PROb tumour cells mixed with BCG). Cyclophosphamide not only depletes Tregs numbers by increasing their susceptibility to apoptosis, but also has a deleterious effect on their function in a murine in vitro study, (Lutsiak et al., 2005).

In patients with ovarian cancer, a direct correlation has been shown between tumor infiltrating Tregs and overall survival. In these patients, treatment with the recombinant interleukin 2 diphtheria toxin conjugate DAB389IL-2 (denileukin diftitox; ONTAK) led to the depletion of Tregs and improved antitumor responses, (Barnett et al., 2005).

In conclusions, a relationship exists between Tregs and the development and progression of lymphoma. These findings indicate the potential importance of Tregs in modifying immune responses in patients with lymphomas. Large scale studies are necessary to confirm these findings, which may provide basis for new anti-lymphoma strategies involving interference in Tregs biology or depletion of Tregs.

References


