Immunostimulation During and After R-CHOP Therapy for B-Cell Lymphoma

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Abstract

Backgrounds: Many studies show an immune imbalance in the tumor environment; some reports show that the T helper 1 (Th1)/ T helper 2 (Th2) ratio, the number of regulatory T-cells (Treg cells) or CD8+T-cells, and the CD8+T-cell/Treg cell ratio are associated with tumor suppression and expansion. Additionally, chemotherapy was reported to affect the immunity of patients with malignancy.

Patients and Methods: Using flow cytometry we measured peripheral blood lymphocytes including non T-cells, as well as T-cell subsets such as CD3+T-cells, CD4+T-cells, CD8+T-cells, Treg cells, Th1 cells and Th2 cells before treatment, at the fourth cycle, and at 1, 3, 6 and 12 months after treatment in 21 patients with B-cell lymphoma receiving R-CHOP therapy. We also analyzed the changes in three immune indexes that reflect anti-tumor immunity (the CD4/CD8 ratio, the CD8/Treg ratio and the Th1/Th2 ratio).

Results: Compared to pre-treatment there were significant decreases in the CD4/CD8 ratio between 1 month and 12 months after treatment (p<0.001, for all time points). The CD8/Treg ratio gradually increased with treatment with significant increases observed at 6 months (p=0.009) and 12 months after treatment (p=0.002). The Th1/Th2 ratio showed a significant increase only before 4 cycles of therapy (p=0.007).

Conclusion: Based on the changes in these three immune indexes, we propose that anti-tumor immunity improved after R-CHOP therapy, which enhanced the efficacy of R-CHOP therapy for lymphoma as well as its direct cytotoxic activity.

Keywords: B-cell lymphoma, R-CHOP, CD8, Regulatory T cell, Th1/Th2.

Introduction

Immune cells are involved in the modulation of internal homeostasis at the time of infection, inflammation, allergy and malignant tumor. In particular, T-cells, which constitute the majority of lymphocytes, play a key role in tumor suppression and development. Many studies have reported that the T-cell balance is disturbed in patients with a malignant tumor, and have suggested that an underlying immune imbalance is related to tumor expansion [1-3]. In addition to the numbers of T-cell subsets, immune balances expressed as ratios of T-cell subsets are also important in order to evaluate immune competence [4, 5].

It was reported that CD4+ and CD8+ T-cells infiltrate into tumor sites and that there are good correlations between the numbers of these cells and patient prognosis [1, 4-6]. CD8+ T-cells exert a cytotoxic effect against tumor cells, and the CD4+ T-cell/CD8+...
T-cell (CD4/CD8) ratio thus reflects immunocompetence; this ratio alters under a tumor microenvironment [1,4,5]. The T helper 1 (Th1)/ T helper 2 (Th2) ratio was shown to decrease in many cancers, and improvement in the Th1/ Th2 ratio enhanced anti-tumor immunity and led to a good outcome [2,3]. On the other hand, regulatory T-cells (Treg cells) are known to suppress the host anti-tumor immunity, and contribute to a worse prognosis [7,8]. Thus, it has also been reported that the high CD8+ T-cell/Treg cell (CD8/Treg) ratio is related to the good prognosis of patients with ovarian and cervical cancer [1,8].

Malignant lymphomas are lymphoid tissue-derived tumors, of which B-cell lymphoma is the most frequently observed, which are treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) therapy that targets B-cells. Th2 polarization was observed in untreated patients with diffuse large B-cell lymphoma (DLBCL), and was recovered after CHOP therapy in patients who had achieved complete remission [3]. Chemotherapy with CHOP or R-CHOP therapy has been reported to affect the host immune system including anti-tumor immunity [3,9-11].

Based on these reports, we hypothesized that the chemotherapy used for malignant lymphoma might lead to an improvement in immune suppression of the tumor and that this suppression may be mediated by T-cell subsets. We therefore measured the numbers of T-cell subsets of patients with B-cell lymphoma before and after treatment using flow cytometry and calculated immune indexes such as the CD4/CD8 ratio, the CD8/ Treg ratio, and the Th1/ Th2 ratio.

### Materials and Methods

#### Patients

This study included 21 untreated patients with B-cell lymphoma who received R-CHOP therapy at Gunma University Hospital, and have achieved at least complete response/ unconfirmed according to Cheson’s Criteria [12] and not relapsed during treatment and up to 12 months. This study was approved by the Institutional Review Board of the Gunma University Hospital Ethics Committee, and all patients and controls provided informed consent.

The patients diagnosed with B-cell lymphoma were histologically defined as 15 DLBCL, three follicular lymphoma (FL), two mucosa-associated lymphoid tissues (MALT) and one mantle cell lymphoma (MCL). Their Clinical Stages (CS) were defined according to Ann Arbor classification modified at Cotswolds meeting, and their prognoses were predicted by the International Prognostic Index (IPI), Age Adjusted IPI (AA-IPI), and Follicular Lymphoma IPI (FLIPI).

Pathological diagnosis, clinical stage, and prognostic index are summarized in Table 1. The clinical characteristics of the patients and the 13 healthy controls are summarized in Table 1. We continue to follow these patients and, to date, have observed 5 relapsed patients after 12 months of treatment.

### Table 1: Characteristics of patients with B-cell lymphoma and healthy controls.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients</th>
<th>Healthy controls</th>
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<tbody>
<tr>
<td>Cases (n)</td>
<td>21</td>
<td>13</td>
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<tr>
<td>Median age (range)</td>
<td>66 (37-78)</td>
<td>59 (49-65)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>14/7</td>
<td>6/7</td>
</tr>
<tr>
<td>Cycle (six/eight)</td>
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#### Diagnosis

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<td>DLBCL</td>
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<tr>
<td>FL</td>
<td>3</td>
</tr>
<tr>
<td>MALT</td>
<td>2</td>
</tr>
<tr>
<td>MCL</td>
<td>1</td>
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</table>

#### CS

| I         | 5  |
| II        | 7  |
| III       | 2  |
| IV        | 7  |

#### Prognostic prediction

<table>
<thead>
<tr>
<th>IPI</th>
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<tr>
<td>Low</td>
<td>5</td>
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<td>Low-intermediate</td>
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<tr>
<td>High-intermediate</td>
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<td>High</td>
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<table>
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<tr>
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<table>
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<tr>
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<td>Intermediate</td>
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#### Clinical data

<table>
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<th>Characteristic</th>
<th>Mean (95% CI)</th>
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<tr>
<td>RBC (×10^6/μl)</td>
<td>4.16 (3.86-4.47)</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>12.59 (11.56-13.61)</td>
</tr>
<tr>
<td>Plt (×10^3/μl)</td>
<td>212.76 (173.35-252.17)</td>
</tr>
<tr>
<td>WBC (×10^3/μl)</td>
<td>6.30 (5.59-7.02)</td>
</tr>
</tbody>
</table>

### Chemotherapy

The patients were treated with R-CHOP (rituximab 375 mg/m² on day 2, cyclophosphamide 750 mg/m² on day 1, doxorubicin 50 mg/m² on day 1, vincristine 1.4 mg/m² to a maximum of 2 mg/m² on day 1, and prednisolone 100 mg/body on day 1-5) therapy. Treatment was repeated after 3 weeks for a total of 6 or 8 cycles.
Flow Cytometric Analysis
Peripheral blood lymphocytes and T-cell subsets were measured using the BD FACSCanto™ II flow cytometer and were analyzed with BD FACSDiva™ software (Becton-Dickinson, Franklin, USA). The samples were measured before treatment, at the fourth cycle, and at 1, 3, 6 and 12 months after treatment.

Measurement of CD3+ T-cells, and CD4+ and CD8+ T-cell Subsets
FITC-labelled anti-CD3, PE-Labelled anti-CD4 and Per-CP-labelled anti-CD8 antibodies (Becton-Dickinson) were added to heparinized blood to stain cell surface antigens. T-cells, CD4+ and CD8+ T-cells were determined as CD3+cells, CD3+CD4+CD8-cells, and CD3+CD4-CD8+cells, respectively.

Measurement of Treg Cells
PE-Cy5-labelled anti-human CD4 (Becton-Dickinson), and FITC-labelled anti-human CD25 (Becton-Dickinson) antibodies were added to heparinized blood to stain cell surface antigens. FITC-labelled Mouse IgG1 κ Isotype Control (Becton-Dickinson) and PE-labelled Rat IgG2a κ Isotype Control (e-Bioscience) were used as control antibodies. The cells were then fixed, permeabilized and stained with PE-labeled anti-human Foxp3 (e-Bioscience, San Diego, USA). Treg cells were determined as CD4+CD25+ Foxp3+ cells.

Measurement of Th1 and Th2 Cells
Heparinized peripheral blood (500μl) was added to RPMI 1640 medium (500μl), and ionomycin (Sigma, St Louis, USA) (1μg/ml) was added to all tubes. Subsequently, Brefeldin A (BFA) (Sigma) (10μl/ml) was added to the resting control and activation tubes and phorbol 12-myristate 13-acetate (PMA) (Sigma) (25ng/ml) was added to the activation control and activation tubes. The tubes were incubated at 37°C under 7% CO₂ for 4 hrs, after which 9 ml of FACS Lysing solution (Becton-Dickinson) was added to each tube and the tubes were incubated at room temperature for 10 min and centrifuged. The supernatants were removed, 2 ml of freezing medium (10% dimethyl sulfoxide (DMSO; Sigma) and 1% bovine serum albumin (BSA; Sigma) in phosphate-buffered saline (PBS)) were added to the pellets and the cells were then frozen at -80°C.

Frozen cells were melted in a 37°C water bath. After washing with 0.5% BSA/PBS, approximately 1×10⁶ cells were suspended in 1ml of FACS permeabilizing solution 2 (Becton-Dickinson) and were incubated at 4°C for 10 min, except for the activation control tubes. After washing again, the cells were stained with the following monoclonal antibodies: PE-Cy5-labelled anti-human CD4 antibodies were used to stain cell surface antigens and Fast Immune™ FITC-Labelled anti-human IFN-γ and PE-Labelled anti-human IL-4 (Becton-Dickinson) antibodies were used to stain intracellular cytokines. Negative controls were Fast Immune™ IgG2a FITC/ IgG1 PE isotype control and mouse IgG1 PE control (Becton-Dickinson), and PE-labelled CD69 antibodies were used as an activation marker. The cells were then incubated at 4°C for 30 min, were washed, PBS was added and the cells were measured. Th1 and Th2 cells were determined as CD4+ IFN-γ+ IL-4- cells and CD4+ IFN-γ- IL-4+cells, respectively.

Measurement of Non T-cells
We considered lymphocytes that do not express CD3 as non T-cells. Non T-cells included cells such as B-cells and NK cells. In this study, non T-cells are alternatively referred to as B-cells.

Other Measurements
Complete blood cell counts and differential white blood counts were determined using ADVIA120 (Siemens, Munich, Germany) or XE-5000 (Sysmex, Kobe, Japan). Serum immunoglobulins (IgG, IgA, and IgM), lactate dehydrogenase (LDH), Interleukin-2 receptor (IL-2R) and C-reactive protein (CRP) were measured using LABOSPECT 008 (HITACHI, Tokyo, Japan).

Definition of recovery
The normal range for T-cell subsets and lymphocytes was defined as the 95% confidence interval (CI) in healthy controls. When the mean values were within the normal range after treatment, we judged that the values were recovered.

Statistical Analysis
We performed an independent t-test to compare the data before treatment to those of healthy controls using the IBM SPSS software package ver. 22 (IBM, Armork, USA). We performed Dunnett’s test using SAS software (SAS institute Inc., Cary, NC, USA), and compared the data before treatment to the data of before 4 cycles, and at 1, 3, 6 and 12 months after treatment. P < 0.05 was considered to be statistically significant.

Results
Comparison of Lymphocytes and T-cell Subsets in Patients before Treatment and in Healthy Controls (Table 2)

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>1.29 ± 0.63</td>
<td>1.74 ± 0.33</td>
<td>0.0123</td>
</tr>
<tr>
<td>T-cells</td>
<td>0.82 ± 0.42</td>
<td>1.22 ± 0.36</td>
<td>0.0097</td>
</tr>
<tr>
<td>non-T-cells</td>
<td>0.47 ± 0.29</td>
<td>0.62 ± 0.19</td>
<td>0.1255</td>
</tr>
<tr>
<td>CD4+ T-cells</td>
<td>0.47 ± 0.24</td>
<td>0.82 ± 0.28</td>
<td>0.0009</td>
</tr>
<tr>
<td>CD8+ T-cells</td>
<td>0.23 ± 0.14</td>
<td>0.29 ± 0.10</td>
<td>0.2025</td>
</tr>
<tr>
<td>CD4/ CD8 ratio</td>
<td>2.53 ± 1.37</td>
<td>2.93 ± 0.93</td>
<td>0.3651</td>
</tr>
<tr>
<td>Treg cells</td>
<td>0.020 ± 0.010</td>
<td>0.006 ± 0.004</td>
<td>0.0000</td>
</tr>
<tr>
<td>CD8/ Treg ratio</td>
<td>12.14 ± 7.59</td>
<td>73.30 ± 50.39</td>
<td>0.0001</td>
</tr>
<tr>
<td>Th1 cells</td>
<td>0.11 ± 0.07</td>
<td>0.13 ± 0.08</td>
<td>0.6724</td>
</tr>
<tr>
<td>Th2 cells</td>
<td>0.012 ± 0.010</td>
<td>0.010 ± 0.012</td>
<td>0.5700</td>
</tr>
<tr>
<td>Th1/Th2 ratio</td>
<td>9.88 ± 5.36</td>
<td>42.76 ± 57.34</td>
<td>0.0252</td>
</tr>
</tbody>
</table>

Table 2: Comparison of T-cell subsets between patients before treatment and healthy controls.

Lymphocytes and T-cell subsets were compared between patients before treatment and healthy controls. The numbers of lymphocytes, T-cells, CD4+ T-cells, and the CD8/Tregs ratio in patients before treatment were significantly decreased compared with healthy controls (p=0.0106, 0.0145, 0.0004 and 0.0013, respectively). The number of Tregs was significantly increased compared with that of healthy controls (p=0.0002). There was no
significant difference between the numbers or ratios of the other lymphocyte subsets between the patients before treatment and the healthy controls.

**Lymphocytes, T-cells, and Non T-cells during and After the Treatment (Figure 1A)**

The numbers of lymphocytes, T-cells and non T-cells were all significantly decreased before 4 cycles of treatment compared with the pretreatment numbers (p<0.0009, 0.0463, and p<0.0001, respectively). After the fourth cycle, the numbers of lymphocytes and T-cells gradually increased. The number of these cells did not recover within normal range after treatment. On the other hand, the numbers of non T-cells remained at significantly lower levels at 1, 3 and 6 months after treatment (p=0.0003, 0.0007 and 0.0036, respectively), but increased at 12 months after treatment.

**CD4+ T-cells, CD8+ T-cells, and the CD4/CD8 Ratio During and After the Treatment (Figure 1B, C)**

Compared to pre-treatment levels, the number of CD4+ T-cells was significantly decreased before the fourth cycle, and at 1 and 3 months after treatment (p=0.0005, 0.0010 and 0.0030, respectively). The number of CD4+ T-cells did not recover within normal range after treatment. The number of CD8+ T-cells was significantly increased at 1, 3 and 6 months after treatment (p=0.0236, 0.0020, and 0.0027, respectively). Hence, there was a significant decrease in the CD4/CD8 ratio at all measurement points (p<0.0001) after treatment.

**Treg Cells and the CD8/ Treg Ratio During and After the Treatment (Figure 1D, E)**

Compared to pre-treatment levels, the number of Treg cells was significantly decreased before the fourth cycle and at 1 and 12 months after treatment (p=0.0099, 0.0100 and 0.0110, respectively). The CD8/Treg ratio gradually increased before the fourth cycle, and there was a significant increase in this ratio at 6 and 12 months after treatment (p=0.0092 and 0.0024, respectively). This ratio recovered within normal range after treatment.

**Th1 Cells, Th2 Cells and the Th1/ Th2 Ratio during the Treatment (Figure 1F, G)**

There was no significant change in the number of Th1 cells during the treatment. There was a significant decrease in Th2 cells before the fourth cycle and at 1 and 3 months after treatment compared with before treatment (p<0.0001, 0.0044, and 0.0086, respectively). There was a significant increase in the Th1/ Th2 ratio before the fourth cycle of treatment (p=0.0069). This ratio recovered within almost normal range after treatment.

**Immunoglobulin (IgG, IgA, IgM) Levels (Figure 2A)**

The serum levels of IgG, IgA, and IgM were significantly decreased at all measurement points (p<0.0001) during and after the treatment compared with those before treatment.

**LDH, IL-2R, and CRP Levels (Figure 2B, C, D)**

There was no significant change in the serum level of LDH due to treatment. Compared to pre-treatment, there was a significant decrease in the level of the IL-2R before the fourth cycle and at
1, 3, 6 and 12 months after treatment (p=0.0015, 0.0008, 0.0005, 0.0010, and 0.0011, respectively). There was also a significant decrease in serum CRP levels at 1, 3, 6 and 12 months after treatment compared with before treatment (p=0.0345, 0.0284, 0.0290, and 0.0438, respectively).

**Figure 2:** Changes in the serum level of immunoglobulins, lactate dehydrogenase, interleukin-2 receptor (IL-2R) and C-reactive protein with treatment. Serum levels of the immunoglobulins IgG, IgA, and IgM (2A), of lactate dehydrogenase (LDH) (2B), of Interleukin-2 receptor (IL-2R) (2C) and of C-reactive protein (CRP) (2D) were measured before treatment, at 4 cycles, and at 1, 3, 6 and 12 months after treatment. Statistical significance is shown as *p<0.05 or **p<0.01.

**Discussion**

Recent studies show that an imbalance of immune cells, including macrophages, T cells, and NK cells, is related to the development and prognosis of malignant lymphoma [6,13,14]. In particular, the balance of T cell subsets, such as CD4+CD8, Th1/Th2 and Treg cells, plays a key role in the progression or suppression of B-cell lymphoma [3,10,15]. In addition, chemotherapy affects the immune system in cancer patients. We therefore assessed T cell subsets in B cell lymphoma patients before and after treatment with R-CHOP therapy.

In this study, the number of CD4+T-cells in patients before treatment was significantly lower than that in healthy controls; however there was no significant difference in the number of CD8+T-cells between patients and healthy controls. CD4+T-cells have been reported to be essential for eradication of advanced B-cell lymphoma, a decrease in CD4+ T-cells would therefore contribute to the establishment of an environment that was suitable for lymphoma [16]. Previous reports showed that high levels of CD4+ T cells in biopsy specimens and in peripheral blood are associated with improved outcome of malignant lymphoma [6,10]. In the present study, the number of Treg cells was significantly higher in patients before treatment compared to healthy controls. An increase in Treg cells in peripheral blood and tumor sites has been reported in many cancers [7,8,15,17,18]. Tumor-induced Treg cells inhibit the proliferation and cytokine production (IFN-γ, IL-2, and IL-4) of T-cells, and also reduce the cytotoxic activity and degranulation of CD8+T-cells [15,19-21]. Furthermore, the number of Treg cells is reported to be a predictor of survival of lymphoma [19]. These investigations support the notion that a decrease in CD4+T-cells and an increase in Treg cells are characteristic features of, and poor prognostic factors for B cell lymphoma.

Th2-predominance in the Th1/Th2 ratio has been reported in many cancers including in DLBCL [2,3,9,22,23]. No significant difference in Th1 cytokine levels, including IL-4, IL-6 and IFN-γ levels, in serum between non-Hodgkin lymphoma patients and healthy controls have been reported [24,25]. Similarly, in the present study, there were no significant differences in the number of Th1 and Th2 cells or the Th1/Th2 ratio between the patients and the healthy controls.

The number of T cells significantly decreased after R-CHOP treatment in our patients. It is has been reported that, although the number of T cells did not change after rituximab-monotherapy [26], the number did decrease after R-CHOP therapy [11]. According to this report, we suspect that the decrease in T cells observed in our study was due to the CHOP therapy.

In the present study the number of CD4+T-cells significantly decreased during treatment and had not recovered by 12 months after treatment. A decrease in peripheral CD4+T-cells has been previously reported in patients treated with R-CHOP therapy [11]. In addition to a decrease in CD4+T-cells induced by cyclophosphamide, especially by high-dose cyclophosphamide such as that used in our study, cyclophosphamide has been reported to promote the induction of circulating CD4+T-cells into the tumor site and their differentiation into CD4+ cytotoxic T-cells (CTL) [27,28]. According to these reports, a decrease in CD4+T-cells might be induced by cyclophosphamide and contribute to the enhancement of anti-tumor immunity. Kurokawa et al. reported that the number of CD4+ T-cells had not recovered by 2 years after treatment and our results are consistent with that report [11]. In the present study the number of CD8+T-cells slightly decreased during treatment, and significantly increased at 3 and 6 months after treatment. An increase in the number of CD8+T-cells after R-CHOP therapy has been previously reported [11]. An increment of CD8+ T-cells, proliferation of T-cells and enhancement of cytotoxic activity of CTL were observed with low-dose cyclophosphamide or doxorubicin treatment [21,27,29]. According to these reports, the observed increase in CD8+T-cells might be induced by CHOP therapy. Moreover, the CD4/CD8 ratio was significantly lower at 12 months after treatment compared to pre-treatment, and contributed to the enhancement of anti-tumor immunity.

In our study the number of Treg cells was significantly decreased by treatment and remained at a low level at 12 months after treatment. Doxorubicin has been reported to reduce Treg cells and to suppress...
the increase in Treg cells induced by myeloid derived suppressor cells (MDSC) [21,28,29]. A low dose of cyclophosphamide was also reported to selectively eliminate Treg cells [31]. Therefore, these chemotherapeutic agents can contribute to a reduction in Treg cells and improvement in anti-tumor immunity.

In our study, the CD8/Treg ratio was significantly lower than that of healthy controls before treatment, but was significantly increased by treatment and had recovered by 12 months after treatment. A previous study reported that patients with ovarian cancer who had a high CD8/Treg ratio after treatment, showed significantly longer overall survival [1]. Consequently, we suggest that the increase in the CD8/Treg ratio that we observed reflects the effectiveness of R-CHOP treatment in our study.

The Th1/Th2 ratio significantly increased during treatment in our study. A previous study indicated that CHOP therapy induced Th1 dominance on the Th1/Th2 ratio in DLBCL patients [3]. Th1 cells facilitate the recruitment and activation of macrophages and CTLs, playing a key role in cytotoxicity, therefore Th1 dominance induced by R-CHOP improves and enhances anti-tumor immunity [32]. Indeed, Ubukata H, et al. showed that patients with gastric cancer who had a high Th1/Th2 ratio had longer overall survival after gastric resection than patients with a low ratio [2].

We also observed a significant decrease in non-T-cells, which consisted mostly of B cells, at 6 months after treatment, and their numbers had not recovered by 12 months after treatment. A previous study reported that B-cells markedly decreased in peripheral blood 2 days after single administration of rituximab, and had not recovered to the normal range by 6 months after treatment [26]. Similar to our results, Kurokawa et al. reported that the recovery of B-cells to diagnosis level was observed at 1 year after rituximab treatment [11].

Immunoglobulin levels were significantly decreased by treatment in our study and remained at lower levels at 12 months after treatment. Although immunoglobulin levels have been reported to be maintained or slightly decrease after rituximab monotherapy, they have been shown to be significantly decreased after R-CHOP therapy [11,26]. According to these reports, the reduction in non-T-cells by rituximab and in Th2 cells by CHOP therapy may contribute to the observed decrease in immunoglobulins. Kurokawa, et al. reported that low immunoglobulin levels continued for 2 years; it will therefore be necessary to pay attention to opportunistic infections 1 year after R-CHOP [11].

Conclusion
In conclusion, a decrease in the CD4/CD8 ratio and an increase in the CD8/Treg and Th1/Th2 ratios by R-CHOP treatment might enhance treatment efficacy in addition to the direct cytotoxicity of the chemotherapy.

Acknowledgements
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References
11. Piguet PF, Vesin C, Grau GE, Thompson RC (1993) Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. Cytokine 5: 57-61.


