Immune Function in Newly Diagnosed Children With Malignancy

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Background: Tumor genesis of many pediatric malignancies remains unclear. Data of immune function are lacking at diagnosis. We prospectively analyzed 109 pediatric patients with malignancy at diagnosis.

Methods: Lymphocyte subpopulations were characterized by FACS, TREC-assay, and Immunoscope, cytokines by FACS and ELISA.

Observations: We detected higher values of CD4+ T cells and consecutively shifted CD4+/CD8+ ratio in all patients compared with the control group. In patients with lymphoma, interleukin-2 was upregulated in all subpopulations.

Conclusions: On the basis of these findings an altered immune function could be found in children with different malignancies at diagnosis. Further investigations are necessary to identify tumor-related immune deficiency for novel therapeutic approaches.

Key Words: pediatric, malignancy, T cells, cytokine production, thymic function, immune function

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BACKGROUND

In Germany, 1800 new patients younger than 15 years contract with a malignancy each year. Leukemia is the most common disease (34%) followed by central nervous system tumors (23%) and lymphoma (12%). Prognosis and tumor entities differ significantly from adult tumors. However, even if prognosis and therapy efforts have dramatically improved over the last decades, malignancies are still the major cause for death in this age group.

Tumor genesis of many pediatric tumors remains unclear. A favorable factor for tumor development is known to be immunodeficiency. For tumor surveillance adequate T-cell numbers and repertoire diversity are key elements that have been extensively studied. In addition, cytokines are critical for the effectiveness of that response. Even if many reports exist about immune reconstitution and function during and after intensive chemotherapy, immune escape mechanism, lymphocyte subpopulations and immune functioning in newly diagnosed children with malignancy before the beginning of therapy were investigated.

MATERIALS AND METHODS

Patients

We studied T cell, B cell and natural killer (NK) cell subpopulations as well as cytokine expression in T cells in 109 pediatric patients with malignancies before therapy. Details of the patients are given in Table 1. Fifty-six patients suffered from leukemia (61%), 26 patients had the diagnosis of lymphoma (28%), whereas 27 patients were diagnosed with solid tumor (29%), 19% of these with a brain tumor. In parallel data of 110 age-matched and sex-matched healthy children were obtained.

The study was approved by the Human Subjects Committee of our institution (Study No. 133/04, 9/28/07, 11/25/05, 12/13/04), patients and guardians participating gave informed consent following institutional guidelines in accordance with the Declaration of Helsinki.

Flow Cytometry

Venous blood samples were collected, anticoagulated with heparin, and processed within 24 hours. Subsets of peripheral blood lymphocytes were monitored by flow cytometry. CD3, CD19, and CD16/CD56 were used to identify T, B, and NK cells, respectively. B-cell and T-cell subsets were further characterized by 4-color flow cytometry to measure the expression of CD27, IgG, and IgM (B cells) and CD4, CD8, T-cell receptor (TCR)-z/8, TCR-78, CD45RA, CD45RO, and HLA-DR (T cells). All monoclonal antibodies were purchased from Becton Dickinson (Heidelberg, Germany). Flow cytometry was performed on a FACS Calibur instrument and data were analyzed using CellQuest Software (Becton Dickinson).

The general gating strategies are explained in Figure 1A. Because some patients suffer from a B-lineage malignancy, others from a T-lineage malignancy we used individualized gating strategies to exclude blasts from analysis to get comparable data in each patient cohort (Figs. 1B, C). Absolute numbers of cells were calculated by multiplying the relative proportion of a particular cell population with the absolute number of lymphocytes (obtained by an automatically analyzed differential white blood count obtained on the same day).
Measurement of Plasma Cytokine Concentrations

Plasma cytokine concentrations were measured using standard 96-well plate ELISA kits according to the manufacturer’s instructions [interferon (IFN)-γ, transforming growth factor (TGF)-β, interleukin (IL)-4, and IL-15 Op-tEIA-kits; Becton Dickinson as well as IL-7 and IL-13; Biosource, Camarillo, CA]. Prepared plates were analyzed on a fluorescence plate reader Genius Plus at 450 nm (Tecan, Crailsheim, Germany). All measurements were carried out in triplicates.

Intracellular Cytokine Staining

Leukocytes (1 × 10⁶) in heparinized peripheral blood (minimum 100 μL) were added to 900 μL RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/L penicillin, sodium-pyruvate 1 mM/L, and 100 μg/L streptomycin (Gibco, Eggenstein, Germany). Diluted peripheral blood cells were incubated with phorbolmyristate acetate (10 ng/mL)/ionomycin (500 ng/mL) and cultured for 20 hours at 37°C and 5% CO₂. To block cytokine secretion, Brefeldin (10 μg/mL; Sigma, Taufkirchen, Germany) was added for at least 16 hours. Intracellular cytokine staining was performed as described previously. IL-2, IL-4, IL-5, IL-10, IFN-γ, tumor necrosis factor (TNF)-α, TGF-β, and Ki67 were all purchased from Becton Dickinson, except TGF-β that was purchased from Biosource.

Immunoscope and TRECks

The diversity of the TCR repertoire of peripheral T cells was analyzed by the Immunoscope method described by Pannetier et al. cDNA was amplified in a real-time polymerase chain reaction assay (Applied Biosystems, Darmstadt, Germany) and products were visualized on the 24 V Fisher gene families. Because a normal Immunoscope consists of 5 to 8 bands per family with a Gaussian distribution of TCR fragments sizes, the score and the size distribution were used to assess TCR repertoire.

Quantification of TRECks in isolated peripheral blood mononuclear cells after FICOLL was performed by real-time quantitative polymerase chain reaction by means of the 5’ nuclelease TaqMan assay with an ABI7300 system (Applied Biosystems) as described. Samples were analyzed in triplicate.

Statistical Analysis

The data were analyzed using JMP (version 5.0) software (SAS, Camarillo, CA). The analyzed parameters are expressed as mean ± SEM unless indicated otherwise. The distribution of different parameters between 2 groups was tested for statistical significance using an unpaired Student t test. The distribution between multiple groups was analyzed using repeated measures analysis of variance and the Bonferroni multiple comparison test. P values of <0.05 were regarded as statistically significant.

RESULTS

Immune Parameters in Leukemia Patients

Patients with leukemia (acute lymphoblastic leukemia) had significantly higher leukocyte counts as well as higher lymphocyte counts compared with healthy age-matched individuals as well as with other tumor patients. Consecutively the relative counts of monocyctic cells and neutrophil cells were diminished. Anemia and thrombopenia were found frequently.

In T cells a naïve phenotype predominated, which fits with the control group because of the young age. CD4⁺/CD8⁺ ratio was evaluated (Fig. 2A). TREC values and diversity of the TCR repertoire were within the expected range. B cell counts were increased (P < 0.02).

Cytokine expression showed no significant differences. TGF-β secretion was elevated at diagnosis (Fig. 2B).

Immune Parameters in Lymphoma Patients

In patients with lymphoma (L) T cell compartment was absolutely and relatively increased compared with age-matched individuals (P < 0.002). The memory phenotype dominated accordingly to age. CD4⁺/CD8⁺ ratio was evaluated (Fig. 2A). TREC values and diversity of the TCR repertoire were within the expected range. HLA-DR were upregulated (P = 0.01).

Using intracellular staining, an increase in intracellular IL-2 production in T cells was found in the lymphoma group, which could be confirmed by comparing these values with an age-matched control group (P < 0.01). To

### TABLE 1. Patient Details

<table>
<thead>
<tr>
<th>Sex</th>
<th>Outcome</th>
<th>No. Patients</th>
<th>Median Age (Range) (y)</th>
<th>Male</th>
<th>Female</th>
<th>Alive</th>
<th>Dead</th>
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</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td></td>
<td>56</td>
<td>6.6 (1-16)</td>
<td>33</td>
<td>23</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>B-precursor -ALL</td>
<td>48</td>
<td>5.1</td>
<td>28</td>
<td>20</td>
<td>0</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>T-ALL</td>
<td>2</td>
<td>12.2</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>6</td>
<td>5.6</td>
<td>15</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>26</td>
<td>12.9 (6-17)</td>
<td>7</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MH</td>
<td>17</td>
<td>13.9</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B-NHL</td>
<td>9</td>
<td>12.0</td>
<td>13</td>
<td>14</td>
<td>21</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Solid tumor</td>
<td>27</td>
<td>8.8 (0.7-18)</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Sarcoma</td>
<td>12</td>
<td>13.4</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Neuroblastoma</td>
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<td>4</td>
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<td></td>
</tr>
<tr>
<td>Medulloblastoma</td>
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<td>5.6</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

ALL indicates acute lymphoblastic leukemia; AML, acute myeloid leukemia; MH, Morbus Hodgkin; NHL, non-Hodgkin lymphoma.
FIGURE 1. Gating strategies. A, The lymphocyte population was identified based on sideward scatter (SSC) and forward scatter characteristics (R1). T cells were further defined as CD3-expressing cells in the lymphocyte population (R2). B cells as CD19-expressing cells. CD3⁺ T cells were either analyzed for the expression of CD4 (R3) or CD8 (R4). Interleukin (IL)-2 expression has been analyzed in total CD3⁺ T cells (blue dots) as well as in T-cell subpopulations (IL-2 expression of CD4⁺ T cells marked pink and IL-2 expression of CD8⁺ T cells marked green). B, Because some patients suffer from a B-lineage malignancy, others from a T-lineage malignancy we used individualized gating strategies to exclude blasts from analysis to assure comparable evidence for each patient cohort. Shown is a patient with B-lineage-acute lymphoblastic leukemia. The blasts were characterized by expressing surface markers CD19, CD45, and CD34. We excluded double positive cells for CD34, CD45, and CD19 from further analysis by gating as shown (pink marked blot show gate 4 = R1*R3*R5) and analyzed B and T cells, which did not express characteristic blast markers. C, Gating strategies in a T-lineage leukemia in parallel to (B). The blasts were characterized by expressing surface markers CD3, CD45, and CD34. We excluded double positive cells for CD34, CD45, and CD3 from further analysis by gating as shown and analyzed T cells, which did not express characteristic blast markers. Pinked marked blots show gate 2=R1*R3.
In TGF-β matched control group (compared with other malignancies and with the age-diagnosis (P < 0.01), Fig. 3B). Furthermore there was a significant difference between non-Hodgkin lymphoma (NHL) and Hodgkin disease: NHL patient had a higher IL-2 expression in all T-cell subpopulations (P < 0.01; CD4+ IL-2+: P = 0.02; CD8+ IL-2+: P < 0.001; CD4+ IL-2+: P < 0.03, Fig. 3C). Furthermore there was a significant difference between non-Hodgkin lymphoma (NHL) and Hodgkin disease: NHL patient had a higher IL-2 expression in all T-cell subpopulations (P < 0.002, Fig. 3A). Patients, who are still alive, showed reduced IL-2 levels at diagnosis (P < 0.007, Fig. 3B). TNF-α were likewise slightly overexpressed in CD3+ cells (P < 0.002).

Sera level of cytokines showed no differences.

**Immune Parameters in Tumor Patients**

In tumor patients (T) T-cell, B-cell, and NK-cell distribution was within the normal range. CD4 cells were found to be elevated resulting in an elevated CD4+ /CD8+ ratio (Fig. 2A). TREC values were slightly decreased, whereas the diversity of the TCR repertoire was within the expected range. The activation marker HLA-DR was significantly lower as in a cohort of healthy children (80% ± 70, P < 0.01) or in L or AL patients.

Secretion of cytokines showed no differences between the malignancies for IFN-γ, IL-4, IL-7, IL-13, and IL-15. In TGF-β secretion, values were significantly increased compared with other malignancies and with the age-matched control group (P < 0.05, Fig. 2B).

Among the tumor subgroups we detected a higher CD3+ IFN-γ+ production (P < 0.001) and higher values in the IFN-γ+ELISA (P < 0.0001) in medulloblastoma patients compared with other tumor entities.

**DISCUSSION**

**Lymphocyte Subpopulations**

Although T cell reconstitution after transplantation or intensive chemotherapy has been extensively studied in recent years, data are lacking on immune function in children with malignant diseases before therapy. Recent studies have revealed that immune reconstitution after chemotherapy is accompanied with prolonged B cell and T cell depletion.1,2 However, we hypothesized that already before therapy begins deficits in immune function may exist.

Besides the well-known thrombopenia and anemia in leukemia patients as a result of depressed hematopoiesis we could not observe a reduction in lymphocyte numbers in general. Even if reports exist of decreased B cell and T cell numbers in previously untreated cancer and NHL patients3,7,8 in our cohort only the contribution of CD4 and CD8 were changed. Normal values for CD4+/CD8+ ratio are described to be in between 0.8 and 1.2 age dependently,7 whereas our patients showed mean values between 1.2 and 2.4.

A number of studies looking at immune reconstitution have found a consistent pattern of more rapid recovery in CD8+ cells, resulting in an inverted CD4+/CD8+ ratio shortly after transplantation and intensive chemotherapy.2,9 In the adult setting colorectal cancer patients with a low CD4+/CD8+ ratio at diagnosis are known to have a better clinical course.10 For other tumors and in pediatric malignancies data are lacking for this issue. Our analysis could not confirm a prognostic impact (P = 0.1), this may be contributed to our pediatric setting, where physiological changes of the quantity and quality of T cell subpopulation during childhood are described.7 However, CD4 overexpression seems to be a specific characteristic for untreated malignancies.

The evidence of the importance of HLA-DR expression in lymphocytes as a late activation marker with regard to prognosis differs. For solid tumors 3 levels of HLA-DR expression are described without significance with respect to prognosis.10 Others report an overexpression of HLA-DR in osteosarcoma patients with prognostic relevance.11 However, values were declined (mean in tumor group: 8%) without correlation to prognosis in our tumor group, and we could demonstrate slightly elevated HLA-DR expression in the lymphoma patients.

**Cytokines**

Cytokines and growth factors are powerful modulators of the immune response. Besides their immunoregulatory function, many cytokines possess growth regulation or macrophage deactivating properties.12 Their aberrant expression either by the tumor cells or by the tumor infiltrating lymphocytes confers a selective advantage to the malignancy to grow and suppress the cytotoxic activity of the infiltrating lymphocytes. Therefore, the type and degree of host response are important elements in determining the outcome of interaction and may be used as a prognostic factor. An imbalance in the regulation and expression of TH1 and TH2 cytokines could play an important role in the etiology of tumor genesis.13

TGF-β is a cytokine, which has important immunomodulatory properties, even though the role in malignancy defence remains unclear. For leukemic blasts an expression is described, which could be a possible explanation for an
immune escape mechanism. The described elevated values of TGF-β in children with tumor might be a similar phenomenon, although only a few data exist on this issue and both, high and low levels are described with an improved outcome.

Higher IFN-γ values in medulloblastoma patients compared with other solid tumors have been described previously. IFN-γ is known to upregulate caspase-8, which induces apoptosis in medulloblastoma. This may lead to improved immunologic tumor surveillance and may therefore turn out to be a prognostic factor, as described.

Most remarkable might be the interesting result of the overexpression of IL-2 in all T cell subpopulations of lymphoma patients. This finding may be because of ineffective effector functions against the malignancy, but it may also indicate a simple reactive counterbalance reaction evoked by tumor excess. However, data exist during therapy that an increased serum IL-2 level proved to correlate with a less favorable prognosis. Unfortunately we did not measure IL-2 serum levels. Furthermore, a relationship between tissue expression of IL-2 and CD25 (IL-2-Rα) and (longer) duration of patients survival in B-cell NHL has been described. However, an increased TH1 ratio has been reported before in certain types of cancer and B-cell lymphoma with lower risk, even before therapy.

IL-2 polymorphisms in lymphoma patients have not been study yet. We hypothesize, that this might be of prognostic relevance and might help to identify high-risk versus low-risk patients.

Taken together, this is the first systematic report, which demonstrates an altered immune function in children with malignancy at the time point of diagnosis before start of therapy. These findings might be a step towards a better understanding of tumor dynamic and immune escape mechanism. Further investigations are necessary to determine individualized prognostic settings toward an improved and risk-adapted therapy.

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