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Intracellular cytokine profile of T cells from children with acute lymphoblastic leukemia

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Abstract Purpose: During an ongoing immune response, cytokines produced by T helper types 1 (Th1) and 2 (Th2) together with T cytotoxic types 1 (Tc1) and 2 (Tc2) are critical to the effectiveness of that response. Dysregulated expansion of one or the other subset may contribute to the impaired function of the T-cell-mediated immune system in cancer patients. In the present study we have investigated whether such dysregulation might exist in children with acute lymphoblastic leukemia (ALL). **Methods:** We analyzed 61 blood samples from 45 children with B cell precursor ALL and 16 healthy children. Interleukin(IL)-2, IL-4, and interferon γ (IFNγ) production of their respective purified $CD4^+$ and $CD8^+$ T cells were assessed at the single-cell level by intracellular-cytokine-staining flow cytometry. **Results:** At the time of diagnosis, IL-2-producing cell populations in $CD4^+$ and $CD8^+$ T cells were reduced below the normal range in 31 of 44 (70.5%) and 23 of 38 (60.5%) cases respectively. Similarly, IFNγ-producing cell populations in $CD4^+$ and $CD8^+$ T cells decreased in 17 of 44 (38.6%) and 18 of 38 (47.4%) cases respectively. Conversely cell populations capable of IL-4 production in $CD4^+$ and $CD8^+$ T cell subsets were increased in 13 of 30 (43.3%) and 15 of 30 (50.0%) cases respectively. Therefore, the Th1-to-Th2 and Tc1-to-Tc2 ratios (1.6 ± 2.2 and 7.7 ± 6.7 respectively) were significantly lower in peripheral blood T cells of ALL patients ($n = 30$) than those (6.0 ± 2.9 and 20.1 ± 10.3 respectively) in 15 healthy controls ($P < 0.0001$). Although both $CD45RA^+/CD4^+$ and $CD45RA^+/CD8^+$ cells significantly increased in 43 ALL patients ($P < 0.05$), there existed no apparent correlation between CD45 isoform expression and cytokine (IL-2 and IFNγ) production. Interestingly, the ability to produce both IL-2 and IFNγ was recovered in 8 cases examined, after complete remission had been achieved. **Conclusion:** These observations suggest that, in both $CD4^+$ and $CD8^+$ T cells of ALL patients, there is a dysregulation in the functionality of Th1 (Tc1) and Th2 (Tc2) cells with a gross reduction of Th1 (Tc1) cell populations and an expansion in Th2 (Tc2).

Key words Intracellular cytokine pattern · Th1/Th2 cell · Tc1/Tc2 cell · Acute lymphoblastic leukemia

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Introduction

The protective value of the immune response is highly dependent on the type of cytokines produced by T cells. According to their cytokine profile, these immune responses have been classified into types 1 and 2, respectively regulated by Th1 and Th2 cell subsets of the CD4 T helper cellular population [1, 9, 27]. Similarly, their CD8 counterparts, T cytotoxic types 1 and 2 (Tc1/Tc2) have been described [22]. Th1 and Tc1 cells promote cellular immunity through interleukin(IL)-2 and interferonγ (IFNγ) production. Th2 and Tc2 cells suppress cellular immunity through increased production of IL-4 and IL-10 [6, 12]. Generally the polarization of the immune response into Th1 or Th2 (Tc1 or Tc2) cellular types is not absolute and the ratio of these cells varies according

to the physiological demand and clinical condition. Furthermore, some pathological conditions may arise from an abnormal balance between the production of Th1 and Th2 (Tc1 and Tc2) cytokines. Common examples of these include the predominance of Th2 profiles in children with atopy and asthma, Th1 in pathological pregnancy and acute graft-versus-host disease, gross cytokine production impairment during infectious disease such as human immunodeficiency virus (HIV) infection, and inclination to Th1 in autoimmune diseases [4, 27, 28].

Leukemic patients have impaired function of the immune system in general and of T cells in particular, manifested by decreased delayed-type hypersensitivity, down-regulation of cytotoxic function, and a diminished proliferative response to polyclonal mitogen or specific antigens [18, 32]. Since the functioning of cytokines produced by T cells is quite important, we hypothesized that dysregulated expansion of the type 1 or type 2 subset may contribute to the development of impaired T cell function in leukemic patients.

Although impaired cytokine production has previously been revealed in cancer patients, the studies invariably determined cytokine production from patients' sera or supernatants of stimulated bulk peripheral blood mononuclear cells (PBMC), making it impossible to delineate the T cell subsets responsible [24]. Flow cytometry, with the advent of the recently developed intracellular cytokine staining technique, has made it possible to determine cytokine production at a single-cell level [5, 13, 23]. In the current study, we investigated $CD4^+$ and $CD8^+$ T cells that can produce IL-2, IFNγ, or IL-4 in children with acute lymphoblastic leukemia (ALL). Our results demonstrate a significant decrease in populations of Th1/Tc1 cell subsets capable of IL-2 and IFNγ production and conversely an expansion of IL-4-producing Th2/Tc2 subsets.

Materials and methods

Peripheral blood mononuclear cells

Heparinized peripheral blood was collected from 45 children, 3–14 years of age (mean, 6.5 years), with B cell precursor ALL at the time of diagnosis. All patients were treated on the chemotherapy protocol (Tokai Pediatric Oncology Study Group Treatment Protocol) [15]. At the time of the study, none of the patients had received any treatment. Peripheral blood was also drawn from the patients 6 months after cessation of chemotherapy. Control blood samples were obtained from age-matched 16 healthy children. Both ALL patients and healthy children had been free of clinical signs of infection during the preceding 4 weeks. The study was reviewed and approved by the Human Subjects Committee of Mie University School of Medicine. Informed consent was obtained from all study participants or their parents.

PBMC were prepared by Ficoll-Hypaque density centrifugation (Histopaque-1077, Sigma). Isolated PBMC were washed three times with RPMI-1640 culture medium and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Because it is well known that these separation procedures affect T cells, the following experiments were performed on PBMC separated from leukemic patients and healthy controls in parallel. The cell suspension from ALL patients contained 1%–80% leukemic blasts, as assessed by May-Giemsa morphology and immunophenotypic characterization [16]. None of the T cell antigens (CD3, CD4, and CD8) was expressed on leukemic blasts examined in the present study. Next, CD4- and CD8-rich T cell subsets were obtained from PBMC by positive immunomagnetic separation using Dynabeads M-450 CD4 and CD8 (Dynal, Skoyon, Norway). The purity of all the purified cells obtained in this way was more than 95%.

Surface marker staining

The proportions of B cells, T cells, and their subpopulations were determined in each sample by immunophenotyping by the lyzed whole-blood method, as described elsewhere [7]. The following monoclonal antibodies (mAb) purchased from Becton Dickinson Immunocytometry Systems (Mountain View, Calif.) were used: fluorescein-isothiocyanate(FITC)-conjugated Leu4 (CD3), Leu18 (CD45RA), CALLA (CD10), phycoerythrin(PE)-conjugated Leu3a (CD4), Leu2a (CD8), Leu12 (CD19), and Leu45RO (CD45RO). Stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson) and results were expressed as the percentage of positive cells in sample preparations.

Cell stimulation and determination of cytokine-producing cell populations by flow cytometry

For in vitro cell stimulation, 1×10^6 purified cells (CD4 and CD8 separately) from each subject were suspended in RPMI-1640 culture medium supplemented with 10% FCS in 12×75-mm polystylene round-bottom tubes and incubated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μM ionomycin (Sigma) in the presence of 2 μM monensin (Sigma) for 12 h at 37 °C in an atmosphere of 95% air and 5% CO_2. The following mAb were purchased from Pharmingen Company (San Diego, Calif.): FITC-conjugated anti-(human IFNγ) (IgG1; clone 4S.B3), PE-conjugated anti-(human IL-2) (IgG2a; clone MQ1-17H12), PE-conjugated anti-(human IL-4) (IgG2a; clone 8D4-8), FITC-conjugated isotype-matched control mAb (IgG1; clone R3-34), and PE-conjugated isotype-matched control mAb (IgG2a; clone R35-95). After stimulation with PMA and ionomycin, cells were doubly stained in the following combinations: FITC-conjugated anti-(human IFNγ)/PE-conjugated anti-(human IL-2) and FITC-conjugated anti-(human IFNγ)/PE-conjugated anti-(human IL-4) alongside controls; FITC-conjugated anti-IgG1/PE-conjugated anti-IgG2a. The flow-cytometric intracelluar cytokine staining protocol has previously been described in detail [7, 13]. Briefly, stimulated cells were fixed and permeabilized by use of OrthoPermeaFix (Ortho Diagnostic Systems, Raritan, N.J.) for 45 min at room temperature, followed by nonspecific blocking with phosphate-buffered saline (PBS) supplemented with 5% FCS for 10 min, again at room temperature. Then, to block the binding of anti-cytokine mAb to Fc receptor expressed on activated T cells, cells were pretreated with Leu-11b mAb (CD16; Becton Dickinson) for 30 min at 4 °C. Next, the cells were washed, resuspended in 5% FCS/PBS, and incubated with the above fluorescein-labeled anti-cytokine mAb together with controls for 30 min at 4 °C. Finally, the stained cells were washed and suspended in 400 μl of FACS flow solution for flow-cytometric analysis. Where CD45RO and CD45RA expressions were determined in combination with intracellular cytokine production, cells were stained for surface markers at the same time as the intracellular cytokine staining.

The intensity of fluorescence was analyzed by a FACScan flow cytometer (Becton Dickinson) having preset instrumentation for uniform analysis of each sample. Ten thousand events of gated viable cells were acquired and analyzed by use of CELLQuest software (Becton Dickinson). Dot-plot quadrant statistics were set on the basis of corresponding isotype-matched control antibodies during data analysis such that frequencies of cell populations capable of IL-2, IL-4, and IFNγ production were determined in each sample. Frequencies of cytokine-producing cells were calculated as follows: (percentage of fluorescent cells stained with anti-cytokine antibody) − (percentage of fluorescent cells stained with isotype-matched control antibody). We defined the cell populations as fol-

lows: populations of $CD4^+$ T cells: Th1, IFNγ-positive and IL-4-negative; Th2, IFNγ-negative and IL-4-positive. Similarly, populations of $CD8^+$ T cells were defined as follows: Tc1, IFNγ positive and IL-4-negative; Tc2, IFNγ-negative and IL-4-positive [5, 7].

Statistical analysis

Student's *t*-test for mean differences was used to analyze data for the levels of statistical significance between ALL patients and healthy controls. In all statistical applications, $P < 0.05$ was considered significant.

Results

Cytokine-producing cells in $CD4^+$ and $CD8^+$ T cell subsets

The $CD4^+$ and $CD8^+$ cell populations capable of IL-2, IL-4 and IFNγ production were assessed by a double-staining intracellular cytokine flow-cytometric technique (Fig. 1). We purified cells prior to stimulation with PMA and ionomycin to determine, with certainty, only cytokine-producing populations of $CD4^+$ and $CD8^+$ T cells without contamination from monocytes and natural killer cells, respectively. More importantly, the aim of this study was to check the preexisting cytokine production capability of the respective T cell subpopulations without any accessory help. To make sure that only intracellular proteins were being quantified, cells were fixed but not permeabilized, giving less than 1.0% fluorescent cells. In addition, preincubating anti-IFNγ antibody with 20 μg/ml human recombinant IFNγ or anti-IL-2 antibody with 20 μg/ml human recombinant IL-2 for 1 h resulted in more than 98% inhibition of fluorescent cells. The percentage of positive cells for intracellular staining in unstimulated culture was routinely less than 1.0%.

In peripheral blood from healthy children ($n = 15$), a large number of $CD4^+$ T cells could produce IL-2 (mean ± SD: 46.7 ± 8.9%) with a smaller but distinct fraction of IFNγ-producing cells (8.6 ± 5.5%). Similarly, the $CD8^+$ T cell population contained a certain number of IFNγ-producing cells (19.6 ± 6.7%) besides those capable of IL-2 production (17.3 ± 6.6%). It is also noteworthy that both $CD4^+$ and $CD8^+$ T cells did contain distinct populations producing both IL-2 and IFNγ. IL-4-producing cell fractions were minimal in both $CD4^+$ and $CD8^+$ T cells in healthy controls (1.5 ± 0.7% and 1.2 ± 0.6% respectively).

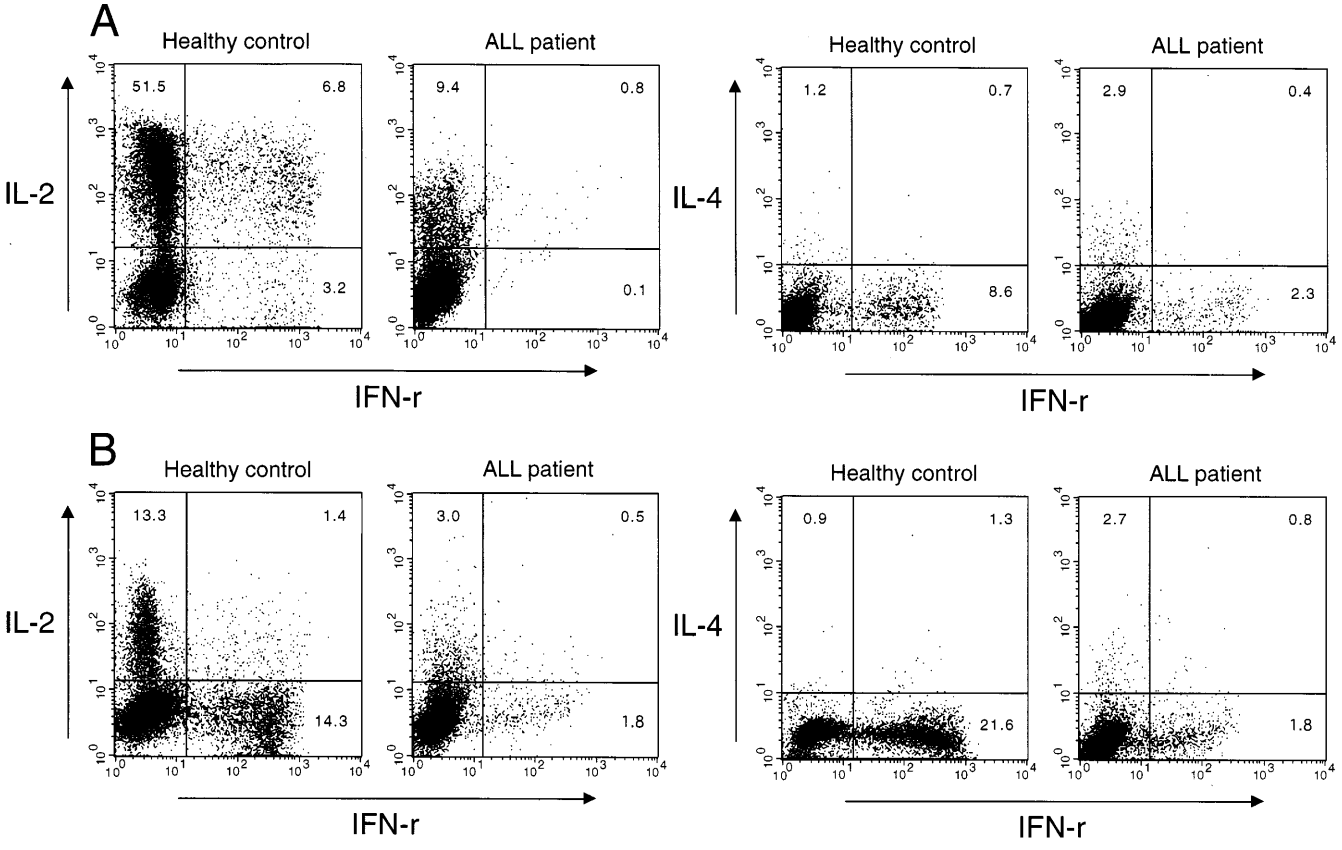

Fig. 1A, B Flow-cytometric analysis of cytokine-producing T cell subsets in peripheral blood obtained from acute lymphoblastic leukemia (*ALL*) patients ($n = 44$) and healthy controls ($n = 15$). $CD4^+$ (**A**) and $CD8^+$ (**B**) cell populations capable of interleukin(*IL*)-2, IL-4, and/or interferon γ (*IFN*γ) production were assessed using dual intracellular cytokine staining and flow cytometry. Representative two-parameter dot plots of IFNγ and IL-2 (IL-4) are shown in dot-plot graphs. Percentages of cytokine-producing cells in each quadrant are indicated. Quadrants of irrelevant-isotype-matched control antibodies had less than 1% fluorescent cells (data not shown). Cell populations capable of producing IL-2 and/or IFNγ are significantly reduced in both $CD4^+$ and $CD8^+$ T cell subsets in ALL patients at the time of diagnosis ($P < 0.05$)

Cytokine-producing cell populations in $CD4^+$ and $CD8^+$ T cells were assessed in 44 children with ALL at the time of diagnosis. Cell populations capable of producing IL-2 were clearly reduced in both $CD4^+$ and $CD8^+$ T cell populations (25.6 ± 15.7% and 9.8 ± 8.3%, respectively) in ALL patients at the time of diagnosis ($P < 0.05$). The percentages of IL-2-producing cells among the $CD4^+$ and $CD8^+$ T cells were reduced below the normal range (mean – SD) in 31 of 44 (70.5%) and 23 of 38 (60.5%) cases respectively (Fig. 2). Similarly, IFNγ-producing cell fractions in both the $CD4^+$ and $CD8^+$ T cell populations (4.8 ± 3.6% and 12.4 ± 8.2% respectively) were distinctly decreased in ALL patients ($P < 0.05$). The percentages of IFNγ-producing cells in $CD4^+$ and $CD8^+$ T cells were reduced in 17 of 44 (38.6%) and 18 of 38 (47.4%) cases respectively. In addition to the reduced fraction of cytokine-producing cells, the fluorescence intensity of intracellular IL-2 or IFNγ staining was lower in leukemic children than that in healthy controls (Fig. 1).

On the other hand, cell populations with the ability to produce IL-4 in both $CD4^+$ and $CD8^+$ T cell subsets (2.6 ± 1.4% and 2.2 ± 1.0% respectively) in ALL patients were larger in healthy controls ($P < 0.05$). The percentages of IL-4-producing cells in $CD4^+$ and $CD8^+$ T cells were increased in 13 of 30 (43.3%) and 15 of 30 (50.0%) cases respectively. The analyses of the ratios of Th1 to Th2 and Tc1 to Tc2 showed that Th1 and Tc1 were dominant in ALL patients. Both Th1-to-Th2 and Tc1-to-Tc2 ratios (Table 1) in ALL patients were significantly lower than those in healthy controls ($P < 0.0001$). We compared the proportions of Th1, Th2, Tc1, and Tc2 cells according to age, gender, leukocyte count, and percentage of leukemic blasts in peripheral blood. However, these patient characteristics did not show any significant difference (data not shown).

In addition, the frequencies of the $CD4^+$ and $CD8^+$ T cell populations with cytokine-producing activity were assessed repeatedly in 8 ALL patients 6 months after cessation of chemotherapy (Fig. 3). The percentage of IL-2-producing cells in $CD4^+$ and $CD8^+$ T cell subsets significantly increased in all cases examined ($P < 0.05$). Similarly, cell populations capable of producing IFNγ in $CD4^+$ and $CD8^+$ T cell subsets clearly increased in 5 of 7 and 5 of 6 cases respectively.

Cytokine production profiles in $CD45RO^+$ and $CD45RA^+$ T cells

Previous studies have shown that $CD45RO^+$ and $CD45RA^+$ T cells differ in their ability to produce

Table 1 Comparison of the Th1-to-Th2 and Tc1-to-Tc2 ratios in acute lymphoblastic leukemia (*ALL*) patients with those in healthy controls. Results are means ± SD

Parameter	ALL patients ($n = 30$)	Healthy controls ($n = 15$)	P
Th1 T cells	4.8 ± 3.6	8.6 ± 5.5	
Th2 T cells	2.5 ± 1.4	1.5 ± 0.7	
Th1-to-Th2 ratio	1.6 ± 2.2	6.0 ± 2.9	< 0.0001
Tc1 T cells	12.4 ± 8.2	19.6 ± 6.7	
Tc2 T cells	2.2 ± 1.0	1.2 ± 0.6	
Tc1-to-Tc2 ratio	7.7 ± 6.7	20.1 ± 10.3	< 0.0001

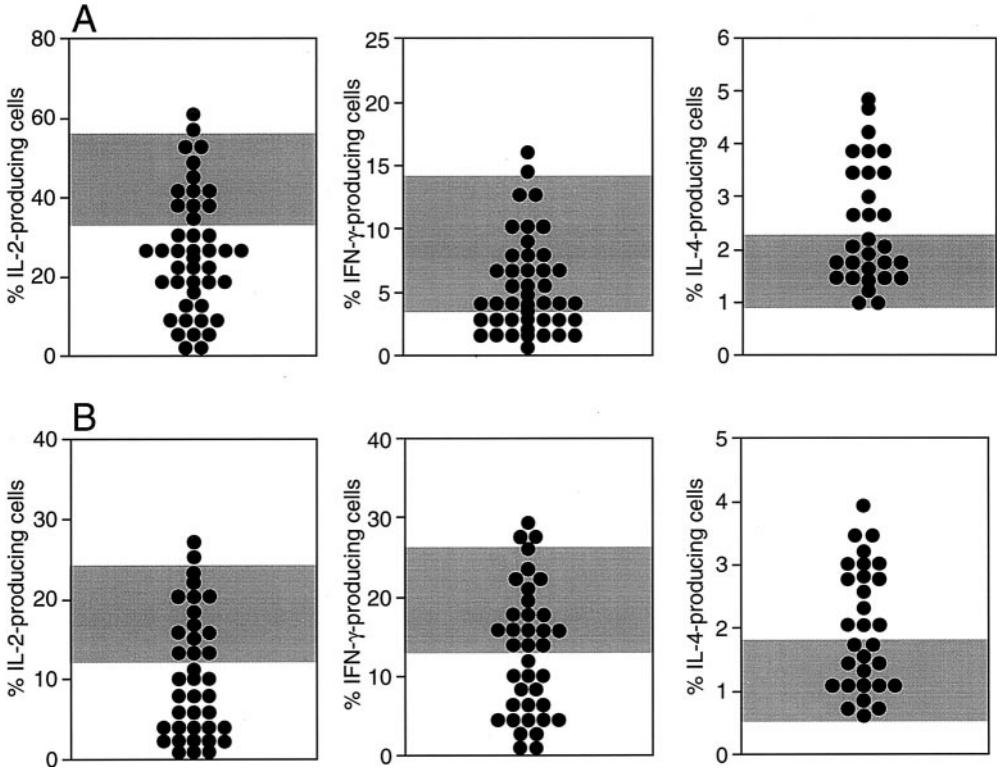

Fig. 2A, B Cells producing cytokines (IL-2, IFNγ, and IL-4) in $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells obtained from ALL patients at onset ($n = 44$). Results were expressed as the percentage of cytokine-producing cells in the $CD4^+$ or $CD8^+$ T cell subset. *Grey areas* means ± SD in healthy controls ($n = 15$). Cell populations capable of producing IL-2 or IFNγ were significantly reduced in both $CD4^+$ and $CD8^+$ T cell subsets in ALL patients at the time of diagnosis ($P < 0.05$). Conversely, cell populations with the ability to produce IL-4 in ALL patients are larger than those in healthy controls ($P < 0.05$)

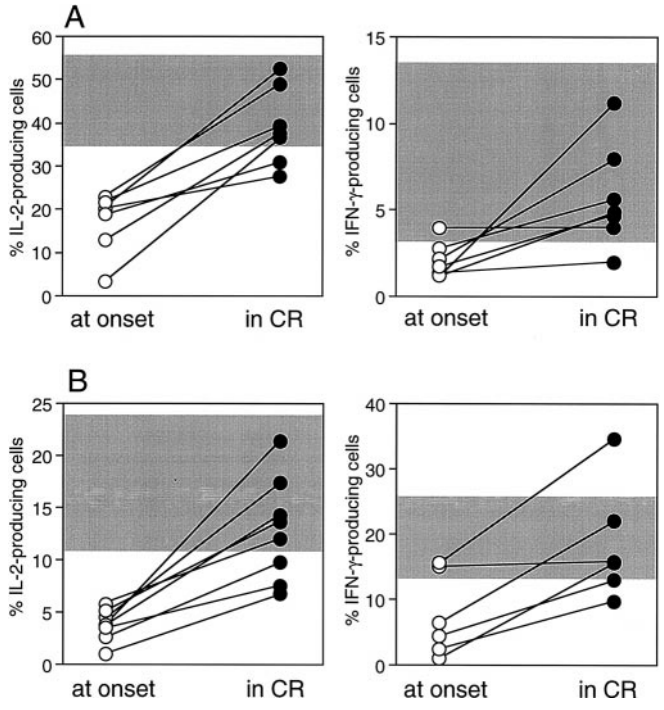

Fig. 3A, B Serial evaluation of cytokine(IL-2 and IFNγ)-producing cells in $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells from ALL patients ($n = 8$) at onset (○) and in complete remission (*CR*) after cessation of chemotherapy (●). Results are expressed as the percentage of cytokine-producing cells in $CD4^+$ or $CD8^+$ T cell subsets. *Grey areas* means ± SD in healthy controls ($n = 15$). The percentages of both IL-2-producing cells and IFNγ-producing cells are significantly increased in both $CD4^+$ and $CD8^+$ T cells from ALL patients in CR ($P < 0.05$)

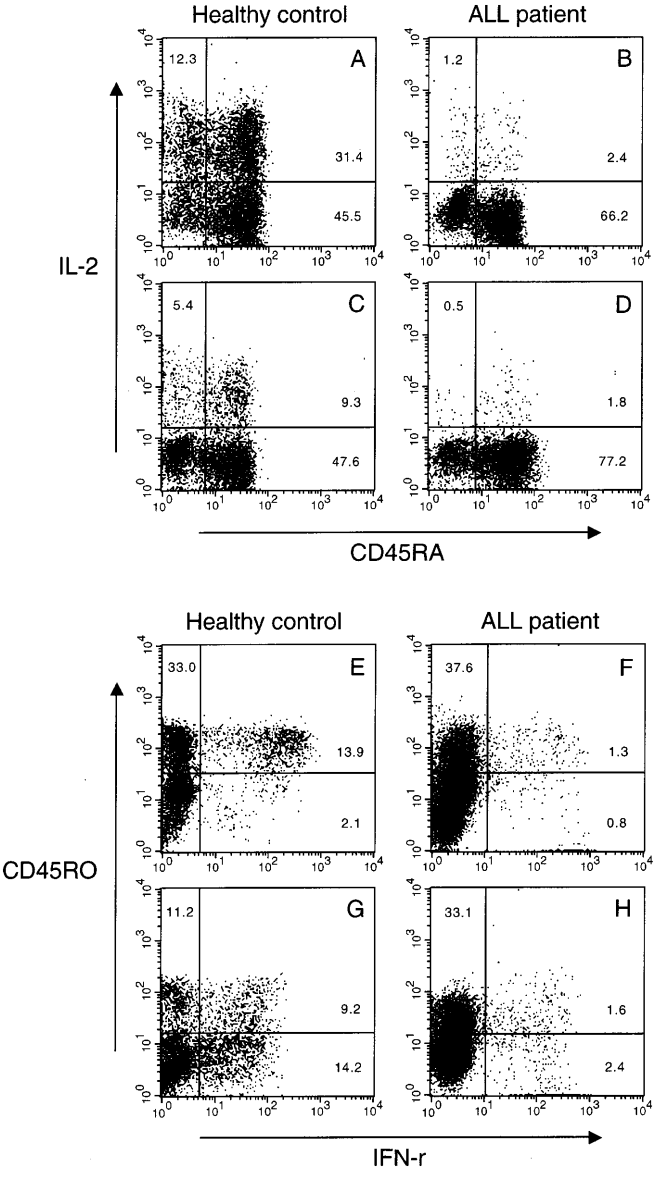

Fig. 4A–H CD45RO and CD45RA expression in IL-2- and IFNγ-producing cells. $CD4^+$ (**A**, **B**, **E**, and **F**) and $CD8^+$ (**C**, **D**, **G**, and **H**) T cells obtained from ALL patients ($n = 15$) and healthy controls ($n = 15$) were stained with anti-CD45 isoform antibodies and anti-cytokine antibodies alongside their respective isotype controls, as described in Materials and methods. Representative two-parameter dot-plots are shown in dot-plot graphs. Percentages of positive cells in each quadrant are indicated. Both IL-2- and IFNγ-producing cell populations are significantly reduced in both $CD45RO^+$ and $CD45RA^+$ T cell subsets ($P < 0.05$)

cytokines [2, 26]. We directly checked the expression of CD45 isoforms in IL-2- and IFNγ-producing cells by flow cytometry (Fig. 4). In healthy children ($n = 15$), almost exclusively, IFNγ-producing $CD4^+$ T cells were $CD45RO^+$. However, only about half of the IFNγ-producing $CD8^+$ T cells were $CD45RO^+$ and a distinct fraction of $CD45RO^-/CD8^+$ T cells could produce IFNγ. IL-2-producing cells have no strong correlation with CD45RA expression. Both $CD45RA^-$ and $CD45RA^+$ cell populations were able to produce IL-2. More interestingly, in ALL patients ($n = 15$), $CD45RO^+/CD4^+$ T cells could not produce IFNγ. Furthermore, IFNγ-producing cell populations were clearly lower in both $CD45RO^-$ and $CD45RO^+$ subsets of $CD8^+$ T cells, than in healthy controls. In addition, IL-2-producing cell populations were reduced in both $CD45RA^-$ and $CD45RA^+$ cell subsets.

Since both $CD45RA^+/CD4^+$ and $CD45RA^+/CD8^+$ T cell populations (73.2 ± 12.4% and 78.3 ± 12.8% respectively) in ALL patient ($n = 43$) were significantly expanded in comparison with those (65.8 ± 10.0% and 72.4 ± 8.1% respectively) in 15 healthy controls ($P < 0.05$), and $CD45RO^+$ and $CD45RA^+$ T cells differ in their ability to produce cytokines, as described above, it is assumed that the increase of $CD45RA^+$ T cell populations might contribute to the dysregulated expansion of type 1 or type 2 T cells in ALL patients. Thus, we decided to investigate the cytokine-production profiles in $CD45RA^+$ and $CD45RA^+$ T cells in 15 ALL patients who showed a significant decrease ($P < 0.05$) of IL-2- and IFNγ-producing cell populations (Fig. 5). The results indicated that the percentages of IL-2-producing cells were significantly reduced in both $CD45RO^+$ and $CD45RA^+$ cell fractions ($P < 0.05$). Similarly, the percentages of IFNγ-producing cells in both $CD45RO^+$ and $CD45RA^+$ cell fractions were

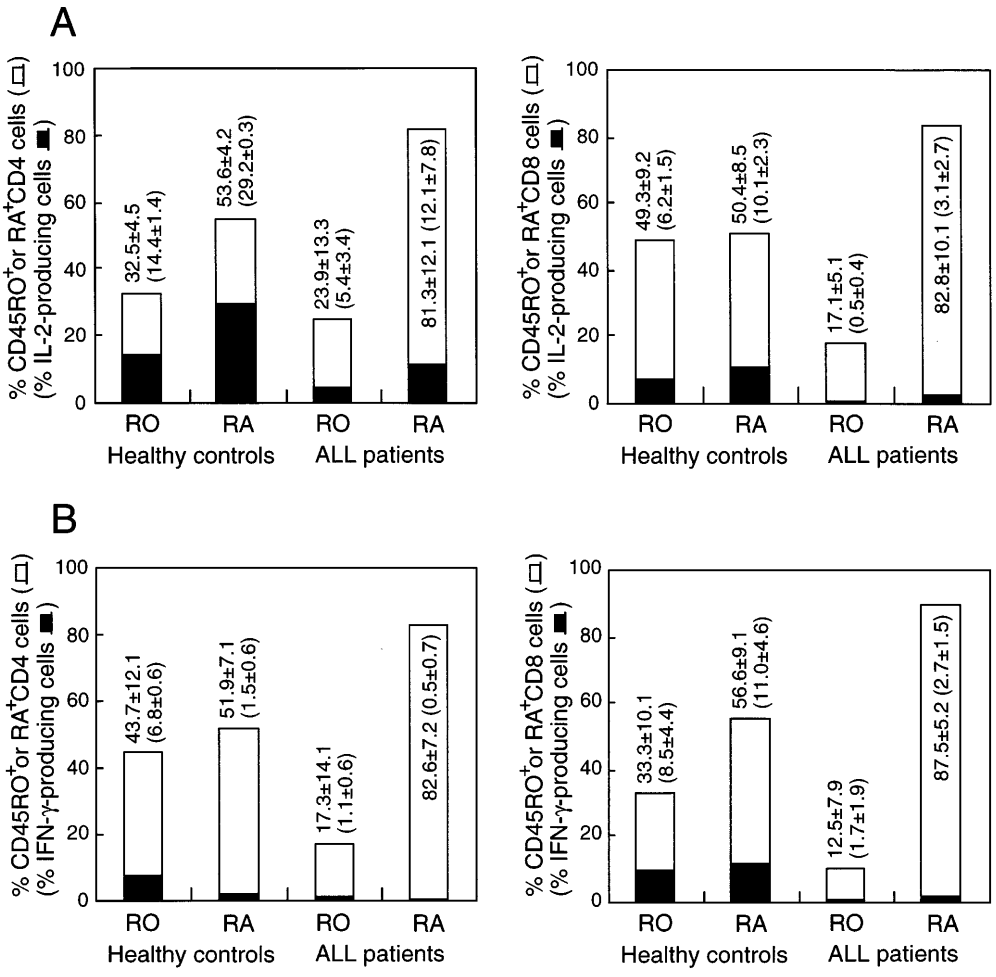

Fig. 5A, B Cytokine-producing (**A** IL-2, **B** IFNγ) cells in $CD45RO^+$ or $CD45RA^+$ T cell populations obtained from ALL patients ($n = 15$) and healthy controls ($n = 15$). The percentages (means ± SD) of $CD45RO^+$ or $CD45RA^+$ cell subsets (*open column*) in the $CD4^+$ or $CD8^+$ T cell population are shown. The percentages (means ± SD) of cytokine producing cell fractions (*closed column*) are also indicated in parentheses. The percentages of IL-2-producing cells are significantly lower in both the $CD45RO^+$ and $CD45RA^+$ cell fractions ($P < 0.05$). Similarly, the percentages of IFNγ-producing cells in both the $CD45RO^+$ and $CD45RA^+$ cell fractions are significantly lower than those in healthy controls ($P < 0.05$)

significantly lower than those in 15 healthy controls ($P < 0.05$). It is of note that no distinct $CD45RA^+/CD4^+$ cell population with the ability to produce IFNγ was identified in either ALL patients or the healthy control groups. Moreover, when individual values for CD45RA or CD45RO expression were plotted on scatter graphs against their respective values for IL-2 or IFNγ production, no significant correlation was demonstrated in $CD4^+$ or $CD8^+$ T cell subsets (data not shown).

Discussion

In the present study, we investigated populations of T cells capable of producing IL-2, IL-4, and IFNγ in ALL children using doubly stained intracellular cytokine flow-cytometric analysis. This technique enables phenotypic characterization of cytokine-producing cells at a single-cell level and therefore allows more accurate and detailed comparison of cytokine profiles in ALL children and healthy controls. The results demonstrated that T cell subsets capable of IL-2 production were reduced in the majority of patients at onset. In addition, IFNγ-producing cell populations also decreased in certain cases. On the other hand, T cell subsets capable of IL-4 production appeared to be expanded in approximately half of the patients, although the percentages of IL-4-producing cells were rather lower than those of IL-2- or IFNγ-producing cells.

The majority of both $CD4^+$ and $CD8^+$ T cells in patients' blood samples express CD45RA antigen indicative of unprimed naive T cells. The percentages of both $CD45RA^+/CD4^+$ and $CD45RA^+/CD8^+$ T cell subsets in ALL children were significantly higher than those in healthy controls. It is now recognized that typical naive T cells invariably produce only IL-2 upon stimulation [5, 7]. Our previous studies consistently demonstrated that $CD45RA^+$ naive T cells in cord blood contained a large number of IL-2-producing cells [7]. However, this is not the case with ALL children, as $CD45RA^+$ T cell subsets in patients appeared to be able to produce neither IL-2 nor IFNγ. Moreover, IL-2-producing cell fractions were reduced in both $CD45RO^+$ and $CD45RA^+$ T cell subsets, indicating that IL-2 production could be impaired not only in unprimed naive T cells but also in memory/effector T cells. We additionally found a reduction of IFNγ-producing cell populations in both $CD4^+$ and $CD8^+$ T cell subsets in certain cases of ALL. Although it has been reported that the $CD45RO^+$ subset accounts for the

preponderance of IFNγ production among both $CD4^+$ and $CD8^+$ T cells [8, 14, 19, 29], the present study does not demonstrate any significant correlation between CD45RO expression and IFNγ production in the respective T cell subsets in ALL patients.

Taking these observations together, it is tempting to hypothesize that the reduced IL-2- and IFNγ-producing cell populations, observed in ALL children, are due to the down-regulation of cytokine protein synthesis in Th1/Tc1 or their precursor cells stimulated with PMA and ionomycin. We previously reported that protein kinase C was normally translocated from the cytosol fraction to the cell membrane fraction in T cells obtained from ALL children at onset [32]. The mobilization of cytoplasmic free calcium was also found to be normal. Thus, the signaling defects in cytokine production systems most likely take effect after protein kinase C activation and calcium mobilization. Recent studies have revealed the impaired activation of NFκB in T cells from a subset of renal cell carcinoma patients, which was mediated by inhibition of phosphorylation and degradation of the inhibitor, IκBα [17]. In addition, both soluble cell extract and culture supernatant from tumor cells inhibited the nuclear translocation of NFκB and degradation of IκBα.

Since NFκB participates in the transcriptional control of a diverse set of genes, such as cytokine genes, the impaired activation of NFκB might be responsible for the suppression of cytokine production in T cells from tumor-bearing hosts. Alternatively, it is possible that the number of Th1/Tc1 or their precursor cells could be reduced in ALL patients. IL-12 and IFNγ are the cytokines most implicated in the expansion of type 1 T cells and in shifting the balance from Th2 (Tc2) to Th1 (Tc1) [10, 20]. Therefore, it is worthwhile investigating IL-12 production from cells of monocyte lineage and the induction of IL-12 receptor expression on T cells in ALL patients. These studies are now in progress in our laboratory.

Various studies have described an essential role for IL-2 in the generation of cytotoxic T lymphocytes (CTL) in both type 1 and type 2 immune responses. IL-2-producing cells are required for the generation and function of mature CTL [21]. Therefore, the result of an IL-2 deficiency might be the suppression of the generation of reactive CTL effector cells, which could allow the cancer to locate and progress in the host. Dysregulation in the functionality of Th1 and Th2 (Tc1 and Tc2) cells with a malfunction in Th1 (Tc1) and an expansion of Th2 (Tc2) may be involved in the basic mechanisms of tumor pathology. In other types of cancer their effects may also be noticed, since the positive resolution of the disease was obtained by therapy inducing Th1 (Tc1) cytokines [11].

Finally we found that the reduction of IL-2 and IFNγ-producing cell populations, observed in ALL children at onset, significantly improved after complete remission was achieved. The elimination of tumor cells, at least, appears to be associated with the reversal of Th1 (Tc1) cytokine production. This observation is consistent with our previous studies, which showed that the suppressed induction of IL-2 receptor expression on activated T cells was corrected following treatment [32]. The decrease of leukemic cell burden might cause the reduced level of inhibitory cytokines or factors possibly released from leukemic blast cells, such as soluble IL-2 receptor [3], transforming growth factor β [30], or IL-10 [25, 31]. Studies are also in progress to clarify whether the culture supernatant of leukemic cells may have any suppressive effect on the functionality of type 1 T cells.

References

1. Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. Nature 383: 787
2. Bass HZ, Yamashita N, Clement LT (1992) Heterogenous mechanisms of human cytotoxic T lymphocyte generation. I. Differential helper cell requirement for the generation of cytotoxic effector cells from $CD8^+$ precursor subpopulations. J Immunol 149: 2489
3. Berghella AM, Pellegrini P, Beato TD, Marini M, Tomei E, Adorno D, Casciani CU (1998) The significance of an increase in soluble interleukin-2 receptor level in colorectal cancer and its biological regulating role in the physiological switching of the immune response cytokine network from TH1 to TH2 and back. Cancer Immunol Immunother 45: 241
4. Byl B, Gerard M, Libin M, Clumeck N, Goldman M, Mascart F (1999) Single cell analysis of antigen-specific T cell response in HIV-infected individuals. Cell Immunol 192: 107
5. Chalmers IMH, Janossy G, Contreras M, Navarrete C (1998) Intracellular cytokine profile of cord and adult blood lymphocytes. Blood 92: 11
6. Chatelain R, Varkila K, Coffman RL (1992) IL-4 induces A Th2 response in Leishmania major-infected mice. J Immunol 148: 1182
7. Chipeta J, Komada Y, Zhang XL, Deguchi T, Sugiyama K, Azuma E, Sakurai M (1998) $CD4^+$ and $CD8^+$ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. Cell Immunol 183: 149
8. Conlon K, Osborne J, Morimoto C, Ortaldo JR, Young HA (1995) Comparison of lymphokine secretion and mRNA expression in the $CD45RA^+$ $CD45RO^+$ subsets of human peripheral blood $CD4^+$ and $CD8^+$ lymphocytes. Eur J Immunol 25: 644
9. Delespesse G, Ohshima Y, Shu U, Yang L-P, Demeure C, Wu C-Y, Byun D-G, Sarfati M (1997) Differentiation of naive human CD4 T cells into TH2/TH1 effectors. Allergol Int 46: 63
10. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM (1993) Development of T TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 260: 547
11. Ito N, Nakamura H, Tanak Y, Ohgi S (1999) Lung carcinoma analysis of T helper type 1 and 2 cells and T cytotoxic type 1 and 2 cells by intracelluar cytokine detection with flow cytometry. Cancer 85: 2359
12. Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, Larner AC, Finbloom DS (1999) Interleukin-10 inhibits expression of both interferon α-and interferon γ-induced genes by suppressing tyrosine phosphorylation of STAT-1. Blood 93: 1456
13. Jung T, Schauer U, Heusser C, Neumann C (1993) Detection of intracellular cytokines by flow cytometry. J Immunol Methods 159: 197
14. Kedl RM, Mescher MF (1998) Qualitative differences between naive and memory T cells make a major contribution to the

more rapid and efficient memory $CD8^+$ T cell response. J Immunol 161: 674
15. Komada Y, Sakurai M (1996) Improved therapy for children with acute lymphoblastic leukemia. Biomed Pharmacother 50: 132
16. Komada Y, Zhou YW, Zhang XL, Azuma E, Sakurai M (1993) Role in growth regulation of cytokines and cytokine receptors in acute lymphoblastic leukaemia expressing myeloid markers. Br J Haematol 84: 408
17. Ling W, Rayman P, Uzzo R, Clark P, Kim HJ, Tubbs R, Novick A, Bukowski R, Hamiton T, Finke J (1998) Impaired activation of NFκB in T cells from a subset of renal cell carcinoma and degradation of the inhibitor, IκBα. Blood 92: 1334
18. Loeffler CM, Smith MJ, Longo DL, Kopp WC, Harvey LK, Tribble HR, Tase JE, Urba WJ, Leonard AS, Young HA, Ochoa AC (1992) Immunoregulation in cancer-bearing hosts: down-regulation of gene expression and cytotoxic function in $CD8^+$ T cells. J Immunol 149: 949
19. Luqman M, Bottomly K (1992) Activation requirements for $CD4^+$ T cells differing in CD45R expression. J Immunol 149: 2300
20. Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S (1993) Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing cells. J Exp Med 177: 199
21. Maraskovsky E, Chen WF, Shortman K (1989) IL-2 and IFNγ are two necessary lymphokines in the development of cytolytic T cells. J Immunol 143: 1210
22. Mosmann TR, Li L (1997) Functions of CD8 T-cell subsets secreting different cytokine patterns. Semin Immunol 9: 87
23. Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A (1995) Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J Exp Med 182: 1357
24. Pellegrini P, Berghella AM, Beato TD, Cicia S, Adorno D, Casciani CU (1996) Dysregulation in Th1 and Th2 subsets of $CD4^+$ T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. Cancer Immunol Immunother 42: 1
25. Perrin GQ, Johnson HM, Subramaniam PS (1999) Mechanism of interleukin-10 inhibition of T-helper cell activation by superantigen at the level of the cell cycle. Blood 93: 208
26. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC (1995) Direct demonstration of cytokine synthesis heterogenity among human memory/effector T cells by flow cytometry. Blood 86: 1408
27. Romagnani S (1994) Lymphokine production by human T cells in disease states. Annu Rev Immunol 12: 227
28. Romagnani S (1996) Short analytical review Th1 and Th2 in human diseases. Clin Immunol Immunopathol 80: 225
29. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer AS, Young HA, Shaw S (1988) Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL-1, CDw29, and Pgp-1) and have enhanced IFNγ production. J Immunol 140: 1401
30. Schiott A, Johansson AC, Widegren B, Sjogren HO, Lindvall M (2000) Effects of transforming growth factor β1 expression in a rat colon carcinoma: growth inhibition, leukocyte infiltration and production of interleukin-10 and tumor necrosis factor α. Cancer Immunol Immunother 48: 579
31. Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, Damme JV, Mantovani A (2000) Autocrine production of IL-10 mediates defective IL-12 production and NF-kappaB activation in tumor-associated macrophages. J Immunol 164: 762
32. Zhang XL, Komada Y, Zhou YW, Chen TX, Sakai H, Azuma E, Ido M, Sakurai M (1997) Inhibition of interleukin-2 receptor (CD25) expression induced on T cells from children with acute lymphoblastic leukemia. Cancer Immunol Immunother 44: 41