Flow Cytometric Detection of Intracellular Cytokines and Chemokines in Acute T Lymphoblastic Leukemia Cells

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Abstract: Intracellular cytokines/chemokines IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-16, IFN-γ, TNF-α, GM-CSF, MCP-1, MCP-3, MIP-1α and RANTES in T lymphocytes from normal donors ($n=8$) and patients with primary acute T lymphoblastic leukemia (T-ALL) ($n=35$) was detected following a 6-hr stimulation with PMA and calcium ionophore A23187. Significantly decreased percentages of cytokine/chemokine positive cells in T-ALL leukemic blasts were observed for IL-2, IL-16, IFN-α, TNF-γ and GM-CSF. In contrast, significantly increased percentage of cytokines/chemokines positive cells in T-ALL leukemic blasts was observed for IL-4, IL-8, IL-12 and MIP-1α. No significant differences were observed between T-ALL leukemic blasts and normal T lymphocytes for IL-1α, IL-3, IL-5, IL-6, IL-10, MCP-1, MCP-3 and RANTES. Also, the T-ALL patients can produce some cytokines/chemokines positive cells that normal donors usually do not produce under the PMA stimulation, such as IL-12 and MIP-1α. In 23 cases of T-ALL patients with the leukemic blast phonotyping of $CD3^-$ $CD7^+$, a minor population of $CD3^+$ $CD7^+$ T cells was also observed in 16 patients. Compared with T lymphocytes from normal donors, the $CD3^+$ $CD7^+$ cell populations in these T-ALL patients revealed significant higher percentages of IL-4, IL-8, IL-12 and MIP-1α cytokines/chemokines positive cells as well as significant lower percentages of IL-2, IL-16, IFN-α, TNF-γ and GM-CSF cytokines/chemokines positive cells. Compared with the $CD3^-$ $CD7^+$ leukemic blasts from these patients, the $CD3^+$ $CD7^+$ cell populations revealed significant higher percentages of IL-2, IL-4 and IL-12, IL-16 and IFN-α cytokines positive cells. No significant differences of IL-1α, IL-3, IL-5, IL-6, IL-8, IL-10, TNF-γ, GM-CSF, MCP-1, MCP-3, MIP-1α and RANTES positive percentages are found between the $CD3^-$ $CD7^+$ and $CD3^+$ $CD7^+$ cell populations. Detection of cytokines/chemokines in leukemic blasts may prove useful for predicting and monitoring response to therapies in which cytokines could be used as potential immunomodulators or therapeutic targets. [Life Science Journal. 2006;3(1):29 - 34] (ISSN: 1097 - 8135).

Keywords: acute T lymphoblastic leukemia; cytokine/chemokine; intracellular staining; flow cytometry

1 Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease resulting from the clonal proliferation of T lymphoid precursors. It accounts for about 15% of all ALL cases in children and 20%-25% in adults (Rivera, 1995; Uckun, 1998). T-ALL is thought to originate inside the thymus and leukemic cells express phenotypic features corresponding to distinct maturational stages of thymocyte development: early (stage Ⅰ), intermediate (stage Ⅱ), or late (stage Ⅲ) (Heerema, 1998; Foa, 1986). Because leukemia cells may retain certain features of their normal counterparts, their characterization with respect to cytokine responsiveness can provide valuable information about the differentiation state of malignant cells and their dependence on the microenvironment. Cytokines/chemokines and its roles in the T-ALL leukemic cells have been studied by many researchers (Dibirdik, 1991; Masuda, 1991; Karawajew, 2000; Scupoli, 2003). It showed the effects of IL-7, IL-4, and IL-2 regarding the induction of proliferation in childhood T-lineage acute lymphoblastic leukemia (T-ALL), and their potential as growth factors has been pointed out (Dibirdik, 1991; Masuda, 1991). Inhibition of *in vitro* spontaneous apoptosis by IL-7 correlates with Bcl-2 up-regulation, cortical/mature immunophenotype, and better early cytoreduction of childhood T-cell acute lymphoblastic leukemia has been observed (Karawajew, 2000; Scupoli, 2003). However, many cytokines/chemokines have not been studied with T-ALL leukemic cells. Therefore, in the current study we investigated 17 different cytokines/chemokines intracellular expression profiles in leukemic blasts as well as in the non-leukemic cells

from 35 patients with T-ALL. Our data demonstrated that many different cytokines/chemokines could be detected in leukemic blasts with intracellular staining by flow cytometry after *in vitro* stimulation. Compared with T lymphocytes from normal donors, a significant decrease of cytokine/chemokine production in T-ALL was observed for IL-2, IL-16, IFN-α, TNF-γ and GM-CSF. In contrast, a significant increase of cytokines/chemokines production in T-ALL was observed for MIP-1α and IL-8. Furthermore, independent analysis of leukemic blasts and non-leukemic cells of T-ALL patients revealed differences in cytokines/chemokines production.

2 Materials and Methods

2.1 Patients and normal donors

All of the 35 patients with T-ALL fulfilled the French-American-British (FAB) Cooperative Group criteria (Bennett, 1981). Age range of patients was 14 – 52 years with 23 males and 12 females. Eight normal donors with age range of 20 – 40 years were included in this study as the normal controls.

2.2 Cells preparation

Peripheral blood samples were collected, after informed consent, from 35 adult patients with newly diagnosed T-ALL. Immunophenotype analysis of T-ALL blasts was performed by direct immunofluorescence and flow cytometry with a FACScan instrument(Becton-Dickinson, San José, CA, USA). Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Hypaque. Before use, the cells' viability consistently exceeded 90% in each sample, as assessed by propidium-iodide (PI) dye exclusion. Then PBMC was stimulated with PMA (phorbol 12-myristate 13-acetate, Sigma) 50 ng/ml and Calcium Ionophore A23187 (Sigma) 1μg/ml in the presence of BD GolgiStop™ protein transport inhibitor 2 mM at 37°C, 7% CO_2 for 6 hours. Stimulated cells were stained surface with CD3 or CD3 and CD7, combining intracellular with anti-cytokine/chemokine monoclonal antibodies (See Table 1). Data was acquired on a FACScan instrument (Becton-Dickinson, San José, CA, USA) and analyzed with CELLQUEST software.

Values were expressed as mean ± SD. Differences (P values) were evaluated using the 2-tailed Student's t-test. Differences were considered statistically significant for $P<0.05$.

Table 1 Anti-cytokine/chemokine monoclonal antibodies used in this study

mAb	Clone name	Format
Il-1α	364-3B3-14	PE*
IL-2	MQ1-17H12	PE
IL-3	BVD3-1F9	PE
IL-4	MP4-25D2	PE
IL-5	JES1-39D10	PE
IL-6	MQ2-13A5	PE
IL-8	G265-8	PE
IL-10	JES3-9D7	PE
IL-12	C11.5	PE
IL-16	14.1	PE
IFN-γ	4S.B3	PE
TNF-α	MAb11	PE
GM-CSF	BVD2-21C11	PE
MCP-1	5D3-F7	PE
MCP-3	9H11	PE
MIP-1α	11A3	PE
RANTES	2D5	PE
CD3	HIT3a	PE-Cy5
CD7	M-T701	FITC**

* PE: Phycoerythrin. ** FITC: Flourescein isothiocyanate. # All monoclonal antibodies are from BD Biosciences Pharmingen.

Table 2. Comparison of cytokine producing cells between normal donor T cells and T-ALL leukemic blasts.

	Normal T lympho (N=8)	T-ALL Blasts (N=35)	*P* Value
IL-1α	0.3±0.1	0.3±0.2	>0.05
IL-2	52.8±7.4	15.5±13.3	<0.001
IL-3	0.5±0.3	0.3±0.2	>0.05
IL-4	3.2±1.3	5.8±5.3	>0.05
IL-5	0.4±0.3	0.4±0.2	>0.05
IL-6	0.5±0.1	0.8±0.7	>0.05
IL-8	9.7±2.6	47.8±33.5	<0.05
IL-10	0.3±0.1	0.4±0.2	>0.05
IL-12	0.2±0.1	2.0±2.4	<0.05
IL-16	91.9±3.8	30.9±36.0	<0.001
IFN-γ	19.8±7.1	8.2±6.1	<0.001
TNF-α	33.2±7.5	10.4±10.4	<0.001
GM-CSF	12.3±4.0	5.2±4.5	<0.01
MCP-1	0.3±0.1	0.3±0.2	>0.05
MCP-3	0.5±0.3	0.4±0.2	>0.05
MIP-1α	4.3±2.1	28.2±23.4	<0.05
RANTES	0.3±0.1	0.3±0.2	>0.05

3 Results

In the T-ALL patients, two different kinds of immunophenotyping of the leukemic blasts were observed in this study. Among these 35 T-ALL patient circulating leukemic blasts, 23 patients' blasts

had the phenotyping of $CD3^-$ $CD7^+$, while 12 patients' blasts had the phenotyping of $CD3^+$ $CD7^+$ (Figure 1). In the 23 patients' blasts with the $CD3^-$ $CD7^+$ phenotyping, a minor $CD3^+$ $CD7^+$ cell population was observed in 16 patients (Figure 2). After staining with different fluorescence conjugated antibodies, intracellular cytokines/chemokines were analyzed in these different cell populations using different flow cytometric gating strategies.

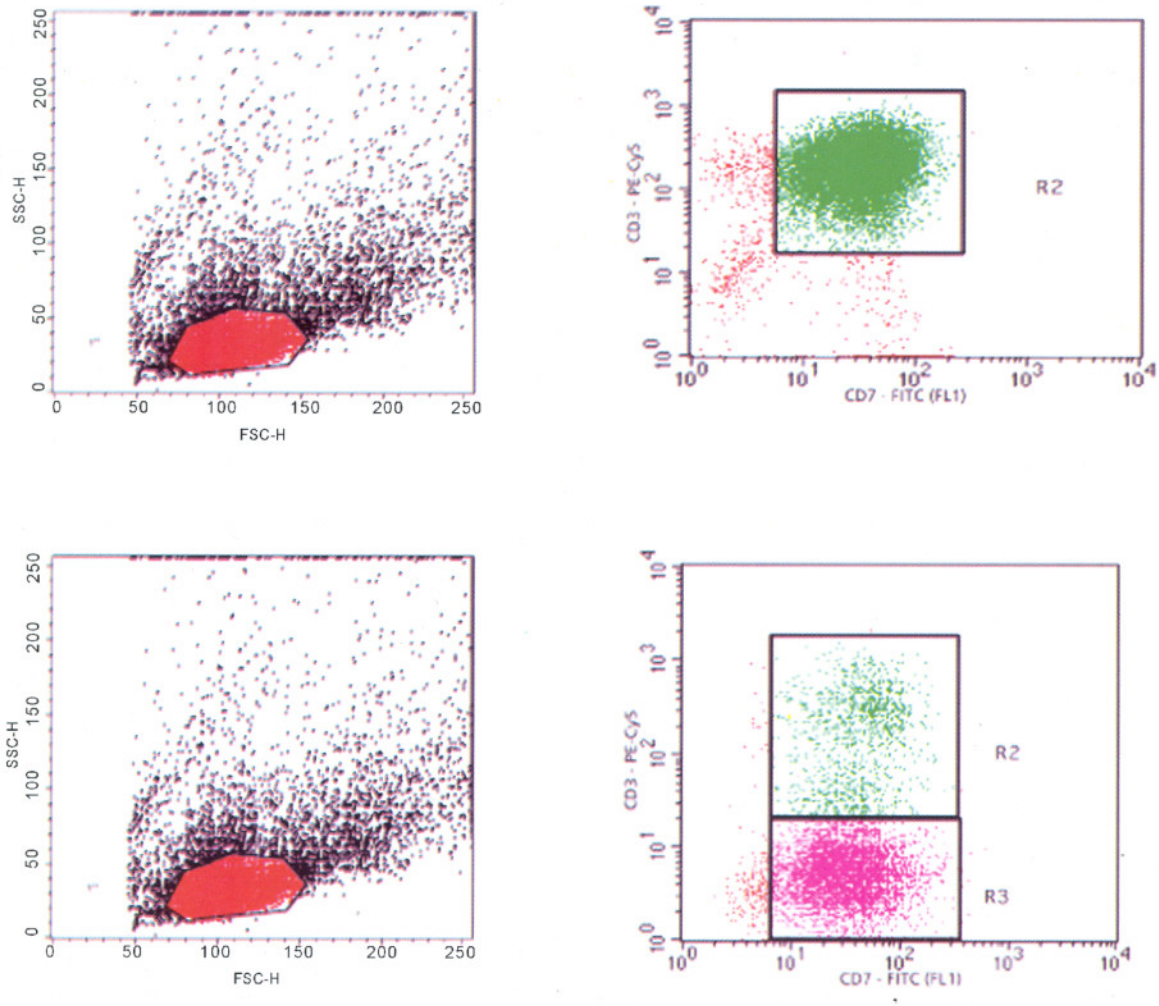

Figure 1. Legend: Two different phenotypings of leukemic blasts were observed in this study, $CD3^+$ $CD7^+$ and $CD3^-$ $CD7^+$. The upper panel showed the leukemic blasts with $CD3^+$ $CD7^+$ gated on the lymphocytic cells population and analysed with CD3 PE-Cy5 (Y-axis) and CD7 FITC (X-axis). The lower panel showed leukemic blasts with $CD3^-$ $CD7^+$ phenotyping.

Leukemic blasts produce many different cytokines/chemokines that can be detected with intracellular staining by flow cytometry. By gating on the leukemic blast populations, we analyzed different cytokines/chemokines production. We found that leukemic blast could produce many different cytokines/chemokines that could be detected with intracellular staining by flow cytometry, although the variation was quite big between different patients. Compared with normal donor T lymphocytes, significant decreased percentages of cytokine/chemokine positive cells in T-ALL leukemic blasts were observed for IL-2, IL-16, IFN-α, TNF-γ and GM-CSF. In contrast, significant increased percentage of cytokines/chemokines positive cells in T-ALL leukemic blasts was observed for IL-4, IL-8, IL-12 and MIP-1α. No significant differences were observed between T-ALL leukemic blasts and normal T lymphocytes for IL-1α, IL-3, IL-5, IL-6, IL-10, MCP-1, MCP-3 and RANTES (Table 2). Also, the cytokines/chemokines profiles from T-ALL patients were quite different from normal donors. Under the PMA stimulation condition, all normal donors produced detectable amount of IL-2, IL-4, IL-16, IFN-γ, TNF, GM-CSF and IL-8 positive cells, but no detectable IL-1α, IL-3,

IL-5, IL-6, IL-10, IL-12, MCP-1, MCP-3 and RANTES positive cells. However, the T-ALL patients could produce some cytokines/chemokines positive cells that normal donors usually do not produce under the PMA stimulation, such as IL-12 and MIP-1α.

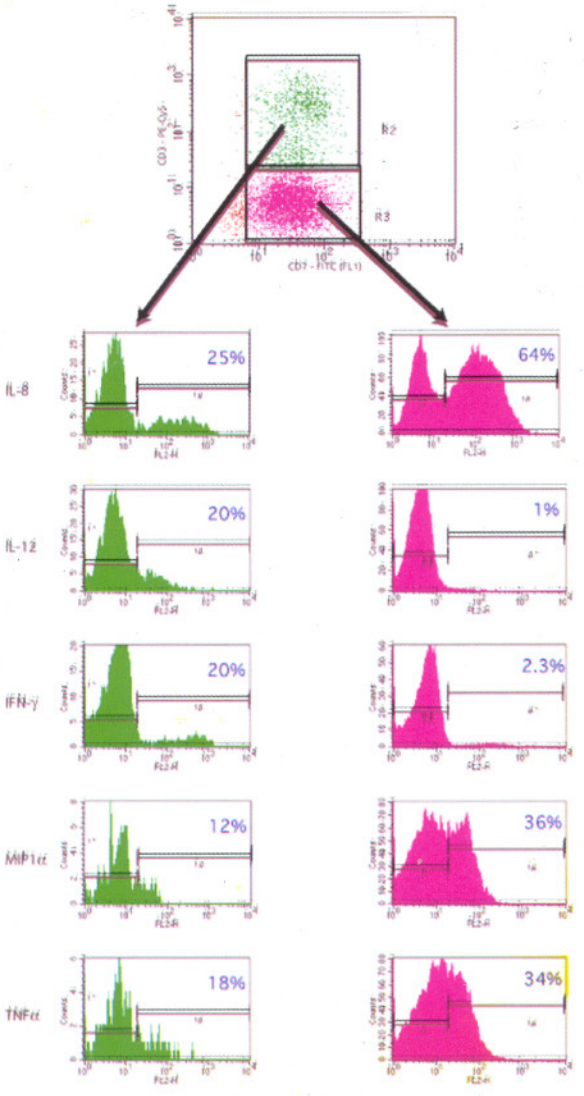

Figure 2. legend: In the 16 patients' blasts with the $CD3^-CD7^+$ phenotyping, a minor $CD3^+CD7^+$ cell population was observed. Independent analysis of the $CD3^+CD7^+$ and $CD3^-CD7^+$ cell populations revealed different profiles in cytokines/chemokines positive cell percentages. These are the typical histograms of different cytokines/chemokines from non-leukemic cells (green) and the leukemic blasts (pink).

In 23 cases of T-ALL patients with the leukemic blast phonotyping of $CD3^-CD7^+$, a minor population of $CD3^+CD7^+$ T cells was also observed in 16 patients. We considered this minor $CD3^+CD7^+$ population as the non-leukemic cells or the normal T cell in these T-ALL patients. Independent analysis of this $CD3^+CD7^+$ T cell population revealed differences in cytokines/chemokines positive cell percentages when compared to either the $CD3^-CD7^+$ leukemic blasts from the same patient or T lymphocytes from normal donors. Compared with T lymphocytes from normal donors, the $CD3^+CD7^+$ cell populations in these T-ALL patients revealed significantly higher percentages of IL-4, IL-8, IL-12 and MIP-1α cytokines/chemokines positive cells as well as significantly lower percentages of IL-2, IL-16, IFN-α, TNF-γ and GM-CSF cytokines/chemokines positive cells (Table 3). Compared with the $CD3^-CD7^+$ leukemic blasts from these patients, the $CD3^+CD7^+$ cell populations revealed significantly higher percentages of IL-2, IL-4 and IL-12, IL-16 and IFN-α cytokines positive cells. No significant differences of IL-1α, IL-3, IL-5, IL-6, IL-8, IL-10, TNF-γ, GM-CSF, MCP-1, MCP-3, MIP-1α and RANTES positive percentages were found between the $CD3^-CD7^+$ and $CD3^+CD7^+$ cell populations (Table 4).

Table 3. Comparison of cytokine producing cells between normal donor T cells and non-leukemic cells ($CD3^+CD7^+$) from T-ALL patients with leukemic blasts of phenotyping $CD3^-CD7^+$.

	Normal T lympho (N=8)	T-ALL $CD3^+CD7^+$ (N=16)	*P* value
IL-1α	0.3±0.1	0.3±0.1	>0.05
IL-2	52.8±7.4	11.6±7.1	<0.001
IL-3	0.5±0.3	0.3±0.1	>0.05
IL-4	3.2±1.3	6.2±4.5	<0.05
IL-5	0.4±0.3	0.4±0.2	>0.05
IL-6	0.5±0.1	0.4±0.2	>0.05
IL-8	9.7±2.6	31.2±19.0	<0.01
IL-10	0.3±0.1	1.0±1.6	>0.05
IL-12	0.2±0.1	7.8±17.1	<0.05
IL-16	91.9±3.8	41.1±30.2	<0.001
IFN-γ	19.8±7.1	9.7±11.7	<0.05
TNF-α	33.2±7.5	8.3±9.5	<0.001
GM-CSF	12.3±4.0	5.4±5.7	<0.05
MCP-1	0.3±0.1	0.9±1.6	>0.05
MCP-3	0.5±0.3	0.3±0.2	>0.05
MIP-1α	4.3±2.1	15.5±13.2	<0.05
RANTES	0.3±0.1	2.7±8.3	>0.05

4 Discussion

Cytokines/chemokines are very important factors in human immuno-regulation. Cytokines/chemokines producing cells detection can provide

critical information for cell-cell interaction in human body. There are different methods to detect cytokines/chemokines producing cells, such as ELISPOT, flow cytometry, immuno-radioactive assay, etc. Intracellular cytokines/chemokines staining by flow cytometry combining with cell surface staining with different monoclonal antibodies not only can detect cytoklines/chemokines producing cells, but also can provide further information such as which of cell subpopulation can produce what kind of cytokines/chemokines. It is known that different cell populations can produce different cytokines. Detection of the cytokines/chemokines producing cells would provide further information for understanding the function of cyteokines/chemokines producing cells in immunoregulation.

Table 4. Comparison of cytokine producing cells between leukemic blasts ($CD3^-$ $CD7^+$) and non-leukemic cells ($CD3^+$ $CD7^+$) from T-ALL patients with leukemic blast phenotyping $CD3^-$ $CD7^+$.

	T-ALL $CD3^+$ $CD7^+$ (n=16)	T-ALL $CD3^-$ $CD7^+$ (n=16)	*P* value
IL-1α	0.3±0.1	0.4±0.2	>0.05
IL-2	11.6±7.1	3.6±7.0	<0.05
IL-3	0.3±0.1	0.5±0.2	>0.05
IL-4	6.2±4.5	3.7±3.2	<0.05
IL-5	0.3±0.2	0.4±0.2	>0.05
IL-6	0.4±0.2	2.4±6.5	>0.05
IL-8	31.2±19.0	39.1±32.2	>0.05
IL-10	1.0±1.6	0.5±0.1	>0.05
IL-12	7.8±17.1	0.6±0.4	<0.05
IL-16	41.1±30.2	19.0±25.2	<0.05
IFN-γ	9.7±11.7	1.0±0.7	<0.05
TNF-α	8.3±9.5	13.0±10	>0.05
GM-CSF	5.4±5.7	7.5±8.6	>0.05
MCP-1	0.9±1.6	1.2±2.5	>0.05
MCP-3	0.3±0.2	0.5±0.2	>0.05
MIP-1α	15.5±13.2	16.6±25.5	>0.05
RANTES	2.7±8.3	0.6±0.5	>0.05

T-ALL is a special type of leukemia involving the T-cell clonal expansion of leukemia cells. We utilized the well-established cytokines/chemokines intracellular staining methods as well as the reagents from BD Biosciences to detect cytokines/chemokines producing cells in T-ALL leukemic patients. The different leukemic blasts not only have different morphology and immunophenotyping, but also have different patterns of cytokine/chemokine producing cells. Although the role of many different cytokines/chemokines in the leukemia still remains mostly unknown, the detection of cytokine/chemokines producing cells both in the leukemic blasts and non-leukemic cells would provide important information regarding the cytokines/chemokines production.

Our data demonstrated the abnormal cytokines/chemokines production pattern in the T-ALL patients, compared with normal T lymphocytes. T-ALL leukemic blasts have the significant lower producing ability for IL-2, IL-16, IFN-α, TNF-γ and GM-CSF. In contrast, T-ALL leukemic blasts have the significant lower producing ability for IL-4, IL-8, IL-12 and MIP-1α. Interestingly, the T-ALL patients can produce some cytokines/chemokines positive cells that normal donors usually do not produce under the PMA stimulation, such as IL-12 and MIP-1α. These abnormalities of cytokine/chemokine production could due to the clonal expansion of the T-lymphoid precursors.

Combined with surface staining of CD3 and CD7 monoclonal antibodies, we further classified the patients' lymphocytes in 16 patients, who have the leukemic blasts with $CD3^-$ $CD7^+$ phenotyping, into two different populations: leukemic blast and non-leukemic cells. The cytokines/chemokines producing cells are different between these two different cell populations. Compared with T lymphocytes from normal donors, the $CD3^+$ $CD7^+$ cell populations in these T-ALL patients revealed significantly higher percentages of IL-4, IL-8, IL-12 and MIP-1α cytokines/chemokines positive cells as well as significant lower percentages of IL-2, IL-16, IFN-α, TNF-γ and GM-CSF cytokines/chemokines positive cells. Compared with the $CD3^-$ $CD7^+$ leukemic blasts from these patients, the $CD3^+$ $CD7^+$ cell populations revealed significantly higher percentages of IL-2, IL-4 and IL-12, IL-16 and IFN-α cytokines positive cells. These cytokines involved both the Th1 and Th2 type cytokines that can be produced by different kinds of T cells. The increase of several different cytokines/chemokines producing cells among the non-leukemic cells in T-ALL patients could be the results of stimulation by the leukemic blasts, or the immuno-regulation of the *in vivo* cell-cell, cell-cytokine interactions. Detection of cytokines/chemokines in leukemic blasts may prove useful for predicting and monitoring response to therapies in which cytokines could be used as potential immunomodulators or therapeutic targets.

5 Conclusions

In this study, we demonstrated that:

(1) Intracellular cytokines/chemokines can be detected in the leukemic cells from T-ALL patients by multiparameter flow cytometry analysis after 6-hour PMA stimulation.

(2) T-ALL leukemic blasts have abnormal cytokine/chemokine producing cell patterns, compared with normal T lymphocytes.

(3) Different levels of cytokines/chemokines productions were observed between the non-leukemic ($CD3^+$ $CD7^+$) and leukemic ($CD3^-CD7^+$) cell populations in T-ALL patients with $CD3^-CD7^+$ phenotyping of blasts.

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