Lymphocytes

Detection of intracellular phosphorylated ERK1/2 in natural killer cells by flow cytometry

At present, immunoblotting, immunoprecipitation and electrophoretic mobility-shift assay (EMSA) are commonly used techniques to study intracellular signaling pathways. In this report, we describe flow cytometric detection of intracellular phosphorylated ERK1/2 and interleukin-15 activation of ERK1/2 in natural killer cell lines. Flow cytometry enables rapid and quantitative assessment of signal protein phosphorylation. Combined with cell surface staining, flow cytometry can discriminate the different activation status of signal proteins in a mixture of cell populations.

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Natural killer (NK) cells are an important component of the innate immune defense.[1] Numerous studies have now convincingly shown that interleukin-15 (IL-15) is a critical factor for NK cell development and function.[2] The interaction of IL-15 with its receptor complex leads to a series of signaling events including the extracellular signal-regulated protein kinases (ERK1/2, p44/42).[3] At present, immunoblotting, immunoprecipitation and electrophoretic mobility-shift assay (EMSA) are commonly used techniques to study intracellular signaling pathways. However, these are all time-consuming, demand a large number of cells and cannot distinguish cell subsets.[4] Flow cytometry has become the technique of choice for quantifying a large number of intracellular molecules involved in signal transduction, cytokine production and so on.[5-9] In this report, we describe IL-15 activation of ERK1/2 in NK cell lines and a flow cytometry technique to detect intracellular phosphorylated ERK1/2.

NK-92 cells were starved of serum and recombinant human (rh) IL-2 for 1 h at 37°C in a 5% CO_2 atmosphere and subsequently stimulated with different doses (0-1000 U/mL) of rhIL-15 for 15 min. After stimulation, 2×10^6 cells were collected, washed in ice-cold PBS and resuspended in 100 μL PBS. Then 100 μL of cold fixation reagent (4°C) were added to the cells for 15 min at room temperature in the dark and followed by incubation of the cells with 500 μL precooled methanol at 4°C for 10 min. After washing in PBS, 100 μL permeabilization medium together with a primary antibody rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (at 1:50 dilution) and anti-p44/42 MAP kinase antibody (at 1:25 dilution), or an isotype control IgG were added to the cells and incubated for 30 min at 4°C in the dark. The cells were then washed twice and resuspended in permeabilization medi-

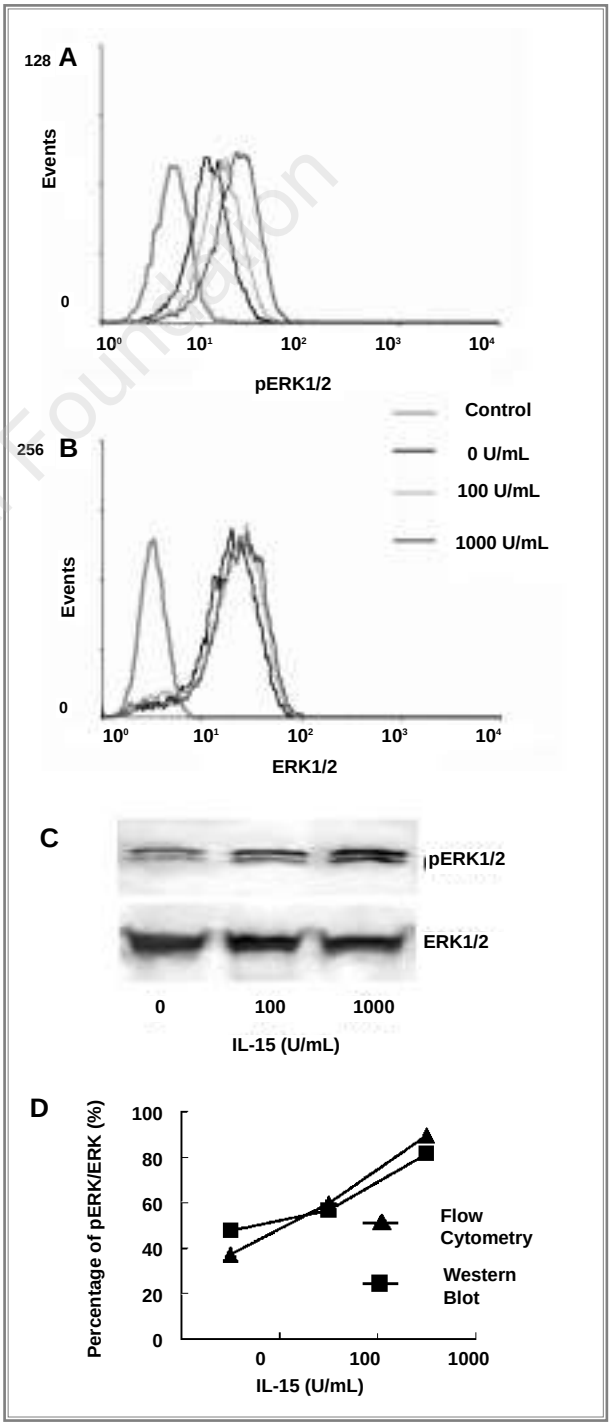

Figure 1. Dose-dependent response of ERK1/2 phosphorylation to IL-15 in NK-92 cells. (A) Detection of ERK1/2 activation by flow cytometry. NK-92 cells were starved of serum and IL-2 for 1 h, incubated with different doses of IL-15 for 15 min, and then permeabilized for intracellular phosphorylated ERK1/2 staining. The results shown are representative of five separate experiments. (B) Detection of total ERK1/2 by flow cytometry. NK-92 cells received the similar treatment as described for (A), but were stained for total ERK1/2. The results shown are representative of three separate experiments. (C) Detection of ERK1/2 activation by Western blot. Serum and IL-2-starved NK-92 cells were stimulated with different doses of IL-15 for 15 min. Protein lysates were made, and the equivalent whole cell extract was evaluated for the presence of phosphorylated and total ERK1/2 protein by Western blot. Similar results were obtained for YT cells (data not shown). (D) The percentage of pERK1/2 versus total ERK1/2 for the same concentration of IL-15. The results shown are representative of three separate experiments performed in NK-92 cells.

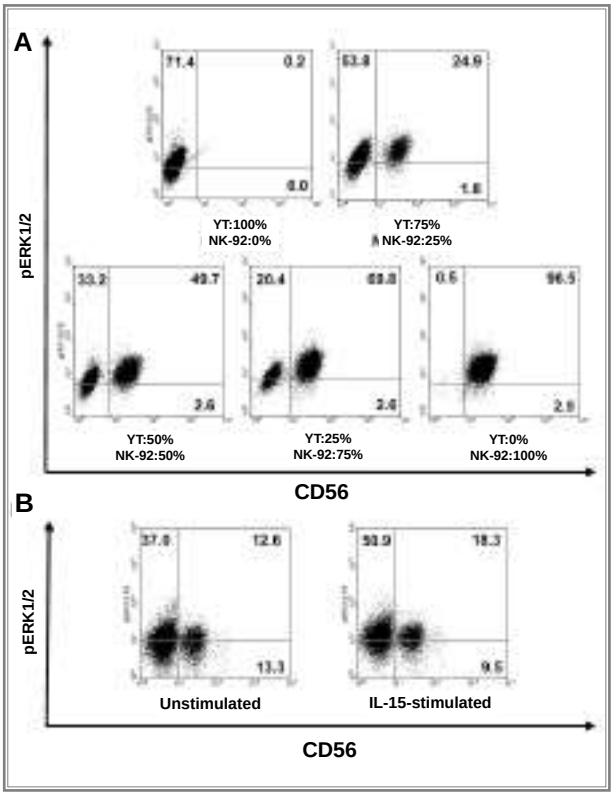

Figure 2. Double-staining analysis of activated-ERK1/2 containing NK cells. (A) After having been deprived of serum and/or IL-2 for 1 h, NK-92 cells were stimulated with 1000 U/mL IL-15 (for 15 min) and then mixed with unstimulated YT cells in different proportions (0-100%), stained with FITC-conjugated CD56 antibody, and also stained for intracellular phosphorylated ERK1/2. (B) Freshly isolated PBMCs were stimulated with 1000 U/mL of IL-15 for 15 min and then stained for surface molecule (CD56) and intracellular phosphorylated ERK1/2. The results shown are representative of three separate experiments.

um with the secondary antibody, PE-conjugated goat anti-rabbit IgG F(ab')$_2$, for 30 min at 4°C in the dark. Following a final wash, the cells were resuspended in 300 μL PBS for flow cytometry. The results are reported as geometric means. Experiments were performed at least 3 times and a representative experiment is shown. Meanwhile, whole cell protein was extracted for Western blotting using a traditional method.

The study of dose-dependent phosphorylation of ERK1/2 in IL-15-exposed NK-92 cells showed that after 15 min of IL-15 stimulation with final concentrations of 0, 100 and 1000 U/mL, ERK1/2 phosphorylation in NK-92 cells increased proportionally to the concentration of IL-15, while the fluorescence intensity of total ERK1/2 was not altered in the culture system regardless of the different doses of IL-15 (Figures 1A and B). Immunoblotting using the same cells showed a similar dose response for phosphorylated and total ERK1/2 (Figure 1C). The percentage of pERK1/2 versus total ERK1/2 for the same concentration of IL-15 is shown in Figure 1D, illustrating that the results obtained from flow cytometry and Western blotting were similar.

The phosphorylation level of ERK1/2 varied with time and when U0126[10] was added to NK-92 cells before IL-15-stimulation, the IL-15-induced ERK1/2 phosphorylation was dose-dependently inhibited (*data not shown*).

We then tried to differentiate NK subsets that had different levels of activation of signal molecules. We examined two human NK cell lines: IL-15-stimulated NK-92 cells which should be CD56$^+$ (surface) pERK1/2$^+$ (intracellular), and unstimulated YT cells which should be CD56$^-$ (surface) pERK1/2^{low} (intracellular). After having been deprived of serum and IL-2, NK-92 cells were stimulated with 1000 U/mL IL-15 for 15 min, while YT cells received no treatment. The two types of cells were then mixed in different proportions (0%-100%). The cell mixture was stained for the surface molecule (CD56) and flow cytometric analysis was performed. As shown in Figure2A, the phosphorylation status of the CD56$^-$ cells (un-induced YT cells) and CD56$^+$ cells (IL-15 stimulated NK-92 cells) was different and the proportion of YT and NK-92 on the double-staining dot blots correlated with the composition of the cell mixture. The cytometric method was used to separate the two NK cell subsets and their phosphorylation status. In addition, we detected the phosphorylation status of ERK1/2 in primary NK cells. Human peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density centrifugation of whole blood from healthy donors using standard procedures. Then PBMC were stimulated with 1000 U/mL IL-15 for 15 min and stained for surface molecule (CD56) and intracellular phosphorylated ERK1/2. As shown in Figure 2B, ERK1/2 was in a phosphorylated state in primary CD56$^+$ NK cells and IL-15-stimulation increased the phosphorylation level.

In conclusion, we found that IL-15 plays an important role in NK cell signaling and, for the first time, describe an alternative method for detecting the intracellular signal molecules, -ERK1/2, of NK cells by double staining flow cytometry.

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Acute Myeloid Leukemia

Patterns of AML1-ETO transcript expression in patients with acute myeloid leukemia and t(8;21) in complete hematologic remission

We used real-time reverse transcriptase polymerase chain reaction (RQ-PCR) to investigate the expression patterns of AML1-ETO fusion transcript during the follow-up of seven patients with acute myeloid leukemia (AML) associated with t(8;21)(q22;q22). In contrast to previous reports of persistent qualitative RT-PCR positivity even during long-term complete remission, our data suggest that a high proportion of t(8;21)-associated AML patients may achieve RQ-PCR negativity after successful treatment. Our data also suggest the possible existence of at least two patterns of transcript expression after successful chemotherapy: 1) early RQ-PCR negativity (sometimes followed by transient positivity); 2) gradual achievement of RQ-PCR negativity.

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The t(8;21)(q22;q22) translocation is found in about 10-15% of patients with acute myeloid leukemia (AML) and is frequently the only cytogenetic abnormality present. It is typically associated with FAB M2 morphology and a relatively good prognosis.[1] The t(8;21) creates an AML1-ETO fusion gene on the derivative chromosome 8. The AML1 gene encodes for a transcription factor essential for normal hematopoiesis, whereas the function of ETO is still unknown.[2,3] Qualitative reverse transcriptase polymerase chain reaction (RT-PCR) detection of residual t(8;21)-positivity has produced contradictory results[4,5] and does not distinguish patients in complete remission (CR) who are cured from those destined to relapse.[6,7] We used real time RT-PCR (RQ-PCR) to investigate expression patterns of AML1-ETO fusion transcript in 68 bone marrow samples taken during the follow-up of seven patients with t(8;21)-associated AML who were routinely addressed to molecular (qualitative RT-PCR) analysis at our Institute from May 1993 onwards (Table 1). Of these, six were treated at the "L. & A. Seràgnoli" Institute, while the remaining patient (#6) was referred to us for molecular monitoring during and after treatment at the Institute of Hematology of the University of Taranto, Italy. All patients provided written informed consent to their participation in the study.

Table 1. Clinical and karyotype characteristics of patients affected by AML with t(8;21)(q22;q22).

Patient No.	*Sex/ Age*	*FAB*	*Therapy*	*Clinical outcome*	*Karyotype*
1	F/31	M2	ICE/NOVIA/BMT	CR/AW	46,XX,t(8;21)(q22;q22)(18)
2	M/47	M1	ICE/NOVIA/ABMT	CR/AW	46,XX,t(8;21)(q22;q22)(12)
3	F/41	M2	ICE/NOVIA/ABMT/FLAN/BMT	2nd CR/AW	46,XX,t(8;21)(q22;q22)(16)
4	F/29	M4Eo	ICE/NOVIA/ABMT-PBSC	CR/AW	46,XX,t(8;21)(q22;q22)(8)/ 46,XX,t(8;21)(q22;q22),der(16), t(3;16)(q21;q22)(13) 46,XX,t(8;21)(q22;q22),del(9)(q22)(4)
5	F/22	M4	ICE/NOVIA/ABMT-PBSC	CR/AW	46,XX,t(8;21)(q22;q22)(20)
6	M/47	M4	FLAI/MEC4	CR/AW	46,XX,t(8;21)(q22;q22)(12)
7	M/22	M2	FLAI/MEC4	CR/AW	46,XX,t(8;21)(q22;q22)(20)
Median (range)	31 (22-47)				

CR: complete remission; AW: alive and well.