## Kinetics of cytokine receptor internalization under steady-state conditions affects growth of neighboring blood cells

Ligand-bound cell surface cytokine receptors are internalized, then either returned to the surface or transferred to lysosomes. The rates of internalization and recycling may vary depending on cell types, ligands, or the environment, and affect cell proliferation, survival, or function. However, the detailed kinetics of this process, particularly in hematopoietic cells, are largely unknown.[1]

A key technical problem is how to discriminate surface receptors from those that are internalized. Conventional methods such as labeling surface receptors with membrane impermeable biotin,[2] dissociating ligands from surface receptors by acid treatment,[3] or morphological analysis using fluorochromes or particles in electron microscopy[3] generally do not work well for hematopoietic cells.

Recently, Johnston *et al.* developed a flow cytometry (FCM)-based specific hybridization internalization probe (SHIP).[4] To measure the internalization and recycling rates of transferrin receptor (TfR; CD71) in hematopoietic cells, they initially labeled cell surface TfR using an antibody fused to a single-stranded oligonucleotide conjugated to a fluorescent dye.[5] The subsequent addition of antisense DNA probes conjugated to a quencher instantly eliminated the fluorescence of surface but not internalized receptors (see *Online Supplementary Materials and Methods*). Using this method, we found that more than 80% of TfR on the surface of erythroblasts (Ter-119$^+$) were internalized within 10 minutes and roughly 60% were recycled (Figure 1A), consistent with previous reports.[5,6]

A notable benefit of SHIP analysis is that it can evaluate lineage-specific receptor kinetics ex vivo by initially measuring internalization/recycling rates of receptors in whole bone marrow cells, then gating cells of a specific lineage according to their surface markers. Taking advantage of this, we measured cKit (CD117) internalization rates in early hematopoietic progenitors (Lin$^-$, cKit$^+$) and found that the internalization rate did not reach 50% after 30 minutes (Figure 1A). We also attempted to measure recycling rates but results were inconsistent (*data not shown*).

Low internalization rates of cytokine receptors were also observed for the interleukin-3 receptor (IL-3R)α chain of murine non-leukemic IL-3-dependent Ba/F3 and FDC-P1 cells growing exponentially in medium containing excess amount of IL-3 (steady-state condition). These cells depended exclusively on IL-3 for proliferation and showed similar growth curves (Figure 1B). IL-3Rα expression on the FDC-P1 cell surface was much higher than that of Ba/F3 cells, with a mild increase after overnight IL-3 starvation (Figure 1C). Both FDC-P1 and Ba/F3 cells displayed >70% of ligand-bound IL-3Rα on the surface

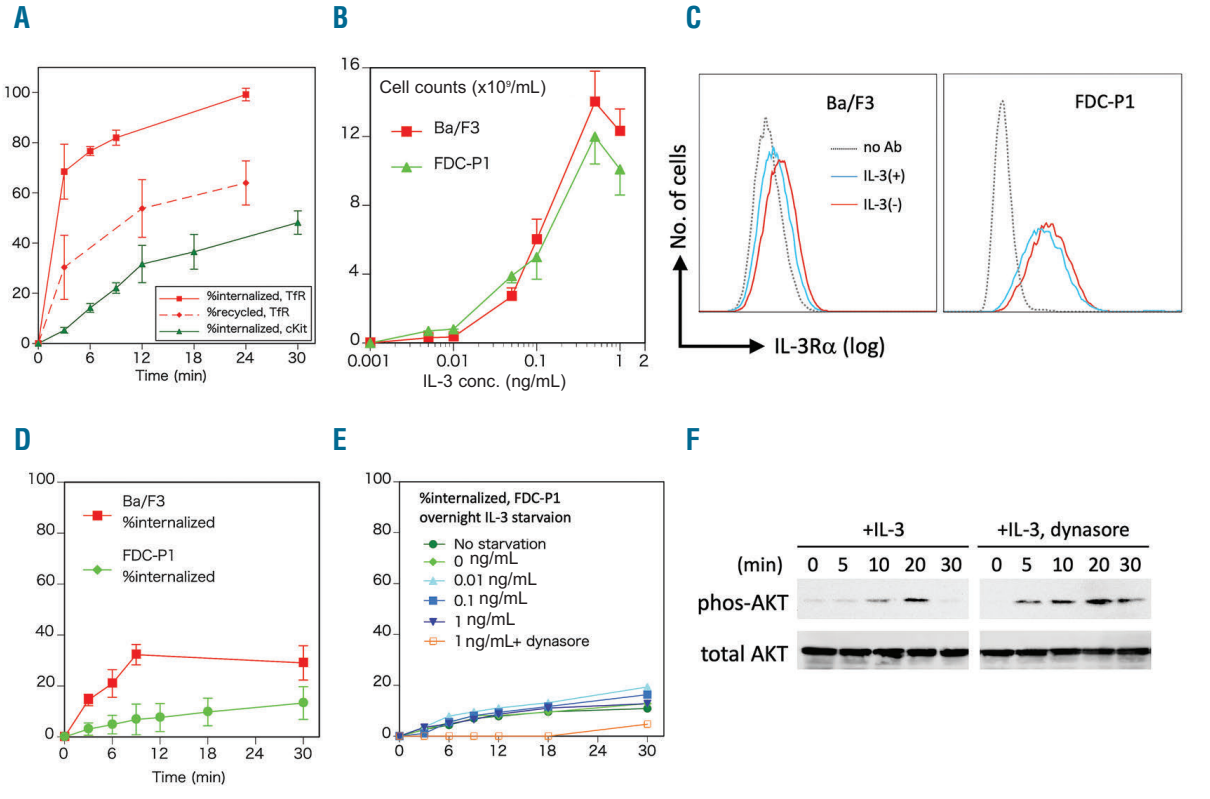


**Figure 1. Relatively low internalization rates of cytokine receptors in hematopoietic cells under steady state conditions.** (A) Specific hybridization internalization probe (SHIP) analysis of transferrin receptor (TfR) internalization (red solid line) and recycling (red dashed line) in bone marrow erythroblasts (Ter-119$^+$) and cKit internalization (green solid line) of hematopoietic progenitors (Lin$^-$cKit$^+$). (B) Growth curves of Ba/F3 and FDC-P1 cells. Cells (1x10$^5$/mL) were cultured for 48 hours in medium containing various concentrations of mouse interleukin (IL)-3. (C) FCM analysis of IL-3 receptor (IL-3R)α expression of Ba/F3 (left panel) and FDC-P1 (right) cells cultured without antibody (negative control), in IL-3 or starved of IL-3 for 6 hours. (D) SHIP analysis of IL-3Rα internalization. Ba/F3 and FDC-P1 cells cultured under steady-state conditions. (E-F) FDCP-1 cells cultured in IL-3–free medium for 16 hours after which IL-3 was added to the culture medium with or without dynasore (25 μg/mL). (E) SHIP analysis of IL-3Rα internalization. (F) Phosphorylated Akt and total Akt were detected by immunoblot analysis. Results are presented as the mean ± standard deviation (SD) of three or more independent experiments (A,B and D); representative data are shown (C-E).

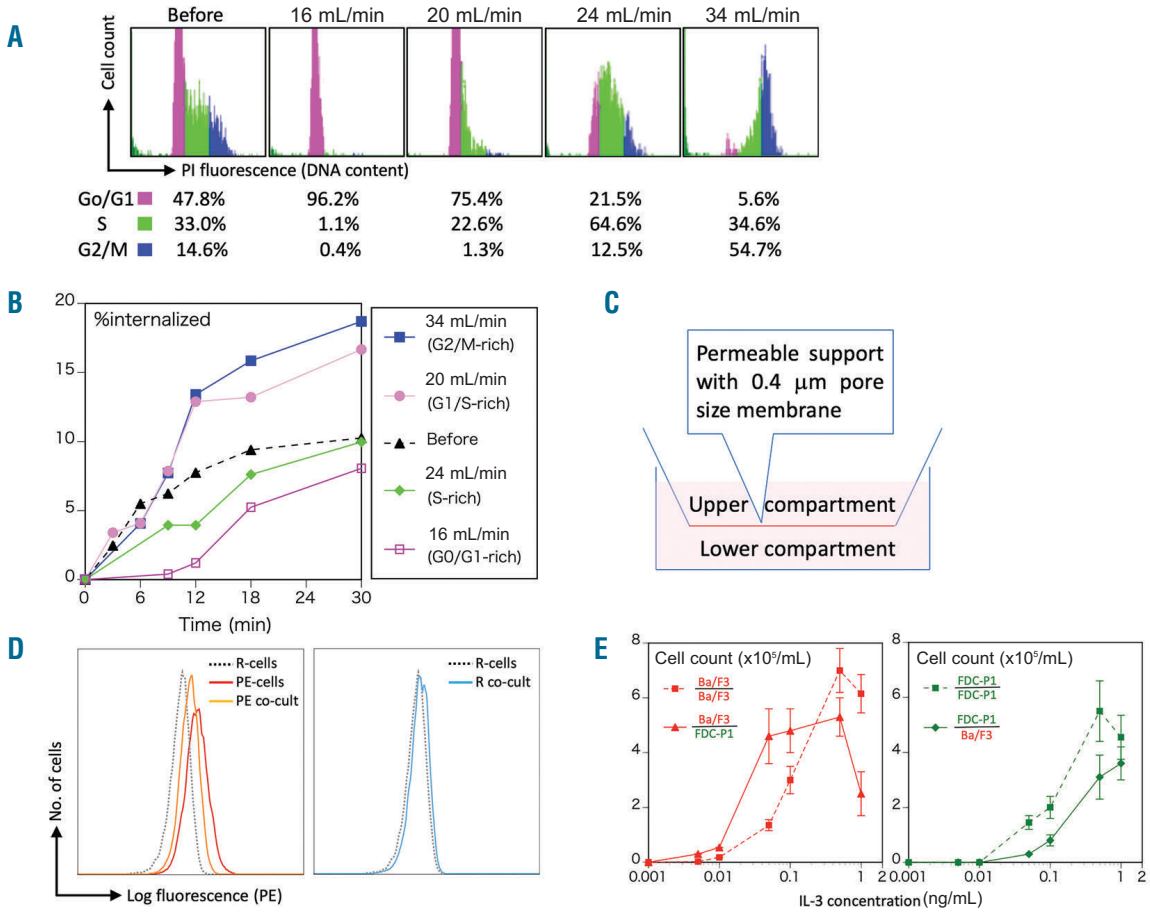


**Figure 2. Biological significance of receptor internalization rates.** (A) Fractions enriched with cells at each phase of the cell cycle were separated by counterflow centrifugal elutriation. Representative DNA histograms are shown of each fraction subjected to flow cytometry after staining DNA with propidium iodide. The percentage of cells in each phase (G1, S, G2/M) is indicated below each histogram. (B) SHIP analysis of IL-3Rα internalization of FDC-P1 cells in each fraction. Representative data are shown. (C) A schema showing a co-culture set-up. (D) FCM analysis of PE- (left) and R-cells (left and right) at the beginning of a co-culture. Two hours later, PE-cells (PE co-culture, left panel) and R-cells (R co-culture, right panel) were subjected to FCM analysis. Representative data are shown. (E) Growth curves of Ba/F3 (left) or FDC-P1 cells (right) co-cultured in upper/lower compartments with FDC-P1 or Ba/F3, respectively, at various IL-3 concentrations. Mean ± standard deviation (SD) of four independent experiments.

for more than 30 minutes (Figure 1D). This indicated FDC-P1 cells had abundant surface receptors showing slow internalization, whereas Ba/F3 cells expressed fewer surface receptors with a relatively high internalization rate under steady-state conditions.

Unexpectedly, the internalization rate of IL-3Rα remained low after prior overnight IL-3 starvation of cells (Figure 1E). This is in sharp contrast to the rapid internalization of other receptors observed in adherent cells. For example, using a biotinylation-based assay we and others demonstrated that surface platelet-derived growth factor receptors (PDGFR) of mouse lung or embryonal fibroblasts that had been starved overnight disappeared almost completely within 20 minutes after the addition of PDGF.[7,8]

This difference could be due to the consequence of receptor internalization. Both ligand-bound surface receptors and internalized (endosomal) receptors are sources of cytokine signals[9,10] but their function likely differs depending on specific cytokines and/or cell types. For example, signals from the surface epidermal growth factor receptor play essential roles in fibroblasts.[11] By contrast, sprouting endothelial cells in the retina have higher rates of vascular endothelial growth factor receptor (VEGFR) endocytosis than cells in more stable and mature vessels, and internalized (endosomal) VEGFR emanates signals necessary for active growth and sprouting.[12] Accordingly, the internalization of ligand-bound receptors may drive cells either to terminate or initiate signaling.

To obtain insight into the consequence of receptor internalization in IL-3-dependent cells, we blocked the internalization of IL-3R using a dynamin inhibitor, dynasore. FDC-P1 cells were IL-3 starved overnight and then treated with dynasore (25 µg/mL) for 30 minutes prior to the addition of IL-3. SHIP assay revealed that IL-3R internalization was blocked completely for >18 minutes (Figure 1E). Immunoblot analysis using an antibody specific for phosphorylated Akt detected increased phosphorylation of Akt in cells treated with dynasore (Figure 1F), suggesting that ligand-bound surface receptors mainly contribute to cytokine signaling.

IL-3 withdrawal causes cell-cycle arrest in the G1 phase followed by rapid apoptosis in IL-3-dependent cells,[13] indicating that cytokine signals are indispensable for cell-cycle progression in the G1 phase. This raises a possibility that cytokine receptor kinetics are affected by the cell-cycle phase. To test this, we performed counterflow cen-

trifugal elutriation to enrich FDC-P1 cells at specific phases of the cell cycle.[14] We obtained four fractions: early G1-, late G1/S-, S-, and G2/M-enriched fractions (Figure 2A). We found that cells in the early G1 phase showed a very low internalizing rate, which was barely detectable at 10 minutes (Figure 2B). By contrast, late G1/S- and G2/M-enriched fractions internalized IL-3Rα more than twice as fast as cells in early G1 and S phase. These findings agree with the above mentioned hypothesis that the internalization of IL-3Rα in IL-3-dependent cells downregulates cytokine signals. We speculate the rapid internalization of PDGFR in PDGF-deprived fibroblasts may also be a way to shutdown excess (unnecessary) signals, because, unlike IL-3-dependent cells, PDGF signals are not necessary for the growth or survival of fibroblasts.

Our findings suggested that ligand-bound cytokine receptors of cytokine-dependent hematopoietic cells exist on the cell surface for a long time. This idea is consistent with the results of co-culture experiments using 0.4 μm pore size membrane inserts permeable to cytokines but that restricted cell transfer (Figure 2C). We initially prepared FDC-P1 cells that had been cultured for 3 hours in medium containing IL-3 conjugated with phycoerythrin (PE-IL-3; PE-cells). PE-cells showed a higher fluorescent signal than FDC-P1 cells that had been cultured with unlabeled IL-3 (R cells; Figure 2D, left panel). PE- and R-cells were then co-cultured in IL-3–free medium. The fluorescent signals of PE cells became reduced after 2 hours (left), while the signals of R-cells increased (right), suggesting PE–IL-3 was released from the surface receptors of PE-cells, according to the dissociation constant, and bound to receptors on neighboring R-cells.

These results suggested that cells such as FDC-P1 expressing abundant surface receptors with a low internalization rate may serve as a cytokine "reservoir" for neighboring cells with receptor metabolism that has fast kinetics. To test this, we performed co-culture experiments with FDC-P1 and Ba/F3 cells. When the same cells were inoculated into upper and lower compartments, the cell growth in each compartment was not significantly different (Figure 2E, dashed lines). The growth of Ba/F3 was accelerated at sub-optimal concentrations of IL-3 (0.05~0.1 ng/mL) by co-culture with FDC-P1 (left panel). By contrast, the growth of FDC-P1 cells was downregulated significantly by co-culture with Ba/F3 cells (right).

It is well known that the biological half-life of cytokines *in vivo* is generally very short (in case of IL-3, only 10-15 minutes).[15] This means that hematopoietic cells *in vivo* are usually under the suboptimal concentration of cytokines, where cytokine internalization rate of neighboring cells is the prime factor to determine cell growth (Figure 2E). Thus our findings depict a new aspect of ligand-bound surface receptors. Cytokines trapped on the surface of cells expressing abundant surface receptors with a slower internalization rate such as FDC-P1 cells, can be re-delivered to surrounding cells and eventually support their growth. In addition, if a cell with abnormally faster kinetics emerges, this would outgrow others by "stealing" cytokines from surrounding cells, leading to its clonal expansion in the bone marrow. This might cause a part of myelodysplastic syndromes, in which, unlike leukemia, pathological cells generally do not show apparent growth advantage. Therefore, we propose that studies on the regulatory mechanisms of cytokine receptor kinetics are critical to elucidate the homeostasis of hematopoiesis and its disruption.

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