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

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Supplemental Materials

Supplemental Methods

Cells and cell culture

Bone marrow cells were separated from 6- to 12-week-old C57Bl/6 mice using a standard procedure. Murine IL-3–dependent Ba/F3 (Riken BRC, Tsukuba, Japan) and FDC-P1 (ATCC, Manassas, VA, USA) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1 ng/mL IL-3 (PeproTech, Rocky Hill, NJ, USA). Co-culture experiments were performed using cell culture inserts with a 0.4 μm diameter (Corning, Oneonta, NY, USA). Cells in an exponential growing phase ($3 \sim 6 \times 10^5$ cells/mL at an IL-3 concentration of 1 ng/mL) were used for experiments unless otherwise indicated. Living cells were counted by trypan blue dye exclusion.

Measurement of receptor internalization and recycling by SHIP analysis

Specific hybridization internalization probe (SHIP) analysis was performed according to the method described by Johnston et al.[1,2] Briefly, (i) *functionalization of antibodies*: antibodies were functionalized using a strained cyclooctyne and kit (Click-IT succinimidyl ester dibenzocyclooctyne alkyne; Life Technologies, Carlsbad, CA) and then coupled with a fluorescence internalization probe (FIP)-azide (5' Cy5-TCAGTTCAGGACCCTCGGCT-N_3 3'; Integrated DNA Technologies, Coralville, IA). (ii) *SHIP internalization assay:* cells were stained on ice for 30 minutes with FIP-Cy5 conjugated antibodies, followed by the incubation of antibody-bound cells at 37°C in serum-free medium to allow the internalization of ligand-bound receptors. Cells were put on ice at specific time-points, resuspended in wash buffer containing propidium iodide (PI) with or without 1 μM quenching probe (QPc: 5' AGCCGAGGGTCCTGAACTGA-BHQ2 3'; Integrated DNA Technologies) and subjected to FCM analysis (FACSCanto II; BD Biosciences, San Jose, CA). (iii) *SHIP recycling assay*: cells were stained on ice for 30 minutes with FIP-Cy5 conjugated antibodies, followed by incubation at 37°C for 6 minutes to allow internalization. Cells were then placed on ice for 5 minutes and 1 μM QPc was added to eliminate fluorescence from surface receptors. After one minute, cells were washed to remove

excess QPc and incubated at 37°C to allow the recycling of receptors inside the cell. Cells were put on ice for specific time-points, stained with PI, with or without 1 μM QPc, and subjected to FCM analysis. (iv) *Gating cells of a specific lineage by surface markers:* Bone marrow cells were stained with fluorescently conjugated antibodies to identify cell lineage at the end of time course just before FCM analysis, in either internalization or recycling assay. Data were analyzed with FlowJo software (BD Biosciences) and internalization/recycling rates were calculated using a formula described in a previously published paper.[2]

Counterflow Centrifugal Elutriations

Counterflow centrifugal elutriations were performed using a SRR6Y elutriation system and rotor equipped with a 4.5-mL chamber (Hitachi Koki Co., Ltd., Tokyo, Japan)[3]. Target cells were resuspended at 1–2 × 10^8 cells in 50 mL of phosphate buffered saline containing 1% fetal bovine serum and injected into the elutriation system at 4°C using an initial flow rate of 16 mL/min and rotor speed of 2,000 rpm. The flow rate was incrementally increased, and cell fractions were collected serially as follows: early G1-enriched fraction, 200 mL at 16 mL/min; late G1/S-enriched fraction, 200 mL at 20 mL/min; S-enriched fraction, 200 mL at 24 mL/min; and G2/M-enriched fraction, 200 mL at 34 mL/min.

Other Experimental Procedures and Reagents

Mouse IL-3 was labeled with PE using a kit (Dojindo, Kumamoto, Japan) according to manufacturer's directions. Dynasore was purchased from Tokyo Chemical Industry (Tokyo, Japan). Antibodies were purchased from the suppliers listed below: mouse TfR (TIB-219) and cKit (2B8) from Bio X Cell (West Lebanon, NH), mouse IL-3Rα (5B11) from BioLegend (San Diego, CA), and phosphorylated Akt (Ser473)(D9E) and total Akt (C67E7) from Cell Signaling Technology (Danvers, MA). For the identification of specific lineages, the following monoclonal antibodies (BD Biosciences) were used: biotinylated Mac-1 (M1/70), Gr-1 (RB6–8C5), B220 (RA3–6B2), CD3 (145–2C11), Ter119, CD16/CD32 (2.4G2; to block Fc receptors), PE-Cy7–conjugated streptavidin and FITC-conjugated Ter119.

Supplemental References

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