Ephrinb1 and Ephrinb2 Are Associated with Interleukin-7 Receptor α and Retard Its Internalization from the Cell Surface*§

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Background: The role of ephrins in regulating IL-7Rα expression was investigated.

Results: Ephrinb1 and ephrinb2 physically interacted with IL-7Rα and regarded its IL-7-induced internalization.

Conclusion: Ephrinb1 and ephrinb2 regulate IL-7R signaling by stabilizing IL-7Rα expression.

Significance: Ephrins could associate with other cell surface molecules and influence the expression of the latter at the post-translational level.

IL-7 plays critical roles in thymocyte development, T cell homeostasis, and the survival of these cells. IL-7 receptor α (IL-7Rα) on thymocytes and T cells is rapidly internalized upon IL-7 ligation. Ephrins (Efns) are cell surface molecules and ligands of the largest receptor kinase family, Eph kinases. We discovered that T cell-specific double gene knock-out (dKO) of Efnb1 and Efnb2 in mice led to reduced IL-7Rα expression in thymocytes and T cells, and that IL-7Rα down-regulation was accelerated in dKO CD4 cells upon IL-7 treatment. On the other hand, Efnb1 and Efnb2 overexpression on T cell lymphoma EL4 cells retarded IL-7Rα down-regulation. dKO T cells manifested compromised STAT5 activation and homeostatic proliferation, an IL-7-dependent process. Fluorescence resonance energy transfer and immunoprecipitation demonstrated that Efnb1 and Efnb2 interacted physically with IL-7Rα. Such interaction likely retarded IL-7Rα internalization, as Efnb1 and Efnb2 were not internalized. Therefore, we revealed a novel function of Efnb1 and Efnb2 in stabilizing IL-7Rα expression at the post-translational level, and a previously unknown modus operandi of Efns in the regulation of expression of other vital cell surface receptors.

IL-7 plays a vital role in thymocyte development (1), T cell homeostatic expansion (2), T cell survival (3), Th1 and Th17 differentiation (4, 5) and, consequently, in various immune responses (6, 7). IL-7 receptors (IL-7R) are composed of IL-7Rα (CD127) and the common γ chain (CD132), which are shared by IL-2R, IL-4R, IL-9R, and IL-15R (1–3). IL-7Rα expression at the mRNA level is suppressed by IL-7 treatment, but needs several hours or even several days to occur. At the post-translational level, IL-7Rα is rapidly internalized within minutes upon IL-7 engagement, and this endocytosis is clathrin-dependent (8). Such regulation at the transcriptional and post-translational levels is likely a negative regulatory loop controlling the strength and duration of IL-7 signaling.

Erythropoietin-producing hepatocellular kinases (Ephs)² are the largest family of cell surface receptor tyrosine kinases, comprising about 25% of known receptor tyrosine kinases (9). A total of 15 Ephs are classified into A and B subfamilies according to their sequence homology; the former has 9 members and the latter, 6, although not all are expressed in a given species (10, 11). The ligands of Ephs, ephrins (Efns), are also cell surface molecules (9). Nine Efns are divided into A and B subfamilies according to the way they anchor to the cell surface. The EfnA subfamily has 6 members that are glycosylphosphatidylinositol-anchored membrane proteins; the EfnB subfamily has 3 members that are transmembrane proteins.

Interactions between Ephs and Efns are promiscuous. One Eph can interact with multiple Efns and vice versa. In general, EphA members preferentially interact with EfnA members, and EphB members with EfnB members (10–12). Such promiscuous interactions indicate that these molecules are so vital to biological systems that heavy redundancy is essential.

Although they are ligands, Efns can also transduce signals into cells (10, 11) in a phenomenon known as “reverse signaling.” Interaction between Eph and Efns results in signaling in both directions, hence, bidirectional signaling. Because Ephs and Efns are both cell surface molecules, they will normally be activated locally by their binding partners expressed on opposing cells during physical contact. Consequently, the major functions of Ephs and Efns are related to pattern formation; how-

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2 The abbreviations used are: Eph, erythropoietin-producing hepatocellular kinase; Efn, ephrin; PE, phycoerythrin; AB, acceptor photobleaching; SE, sensitized emission; CFSE, carboxyfluorescein succinimidy l ester; dKO, double knock-out.
ever, additional functions unrelated to pattern formation have recently been observed.

Most reported functions of Ephs occur in the central nervous system where they are expressed in neurons and control axon and dendrite positioning (10, 11). They are essential in the development of neuronal connections, circuit plasticity, and repair. Some Ephs and Efns also play important roles in other cells and organs, as described below and reviewed by Pasquale (13). They are expressed on endothelial cells and are vital in angiogenesis during normal embryonic development as well as in tumorigenesis. Intestinal epithelial cells express different levels of some Eph and Efnb family members that modulate the movement of epithelial cells along the crypt axis to maintain epithelium self-renewal. Ephb2 and Efnb2 are expressed on the endoderm during embryonic development and their bidirectional interaction is essential in urorectal development. Pancreatic \( \beta \)-cells communicate with each other via Epha and Efnb family members to synchronize their insulin secretion in response to blood glucose fluctuations. Several Ephb and Efnb family members are expressed on osteoclasts and osteoblasts where they regulate bone development, maintenance, and repair. Multiple Eph and Efns have been found to be expressed in some cancer cells and they appear to influence cancer cell growth. Ephb4 and Efnb2 are expressed on hematopoietic progenitor cells and regulate red blood cell production in response to hypoxia. Efnb1 and Epha4 expression in platelets contributes to the clotting process. Efnb1 expression on kidney epithelial cells (podocytes) likely plays a role in glomerular filtration. Interaction between Ephb2 and Efnb2 regulates the ionic homeostasis of vestibular endolymph fluid in the inner ear.

Our group and others have reported that Ephs and Efns, particularly their B family members, as well as some A family members are expressed in thymocytes and T cells; they are capable of modulating T-cell responses and survival (for a comprehensive review on the role of Eph/Efn in the immune system, see Ref. 14). We have shown that Efnb1, Efnb2, and Efnb3 forward signaling through their Eph receptors can co-stimulate peripheral T cells by enhancing cytokine production and proliferation in vitro. We have also demonstrated that one of these Efn receptors, Ephb6, although lacking kinase activity, can transmit signals into T cells, and that its null mutation results in compromised T-cell responses in vitro and in vivo. However, Ephb6 null mutants have normal thymus structure and thymocyte development, probably due to complementary functions of other Eph family members.

In the present study, we discovered that Efnb1 and Efnb2 interact with IL-7R\( \alpha \) on the cell surface and such interaction delays internalization of the latter upon IL-7 stimulation. The significance of this finding is discussed.

**MATERIALS AND METHODS**

**Flow Cytometry**—Single cell suspensions from the thymus, spleen, or lymph nodes as well as T cell lymphoma EL4 and CHO cells were prepared and stained for flow cytometry as described in our previous publication (15). Goat anti-mouse Efnb1, goat anti-mouse Efnb2, and PE-donkey anti-goat IgG Abs were from R & D Systems (Minneapolis, MN). Biotinylated rat monoclonal antibodies (mAbs) in the mouse lineage panel kit as well as the following antibodies (Abs), FITC-mouse anti-mouse CD45.1 (clone A20), APC-rat anti-mouse CD25 (clone PC61), FITC-rat anti-mouse CD25 (clone 7D4), PE-rat anti-mouse CD4 (clones GK1.5 and H129.19), PerCP-rat anti-mouse CD4 (clone RM4–5), biotin-rat anti-mouse CD8b (clone 53–5.8), APC-Cy7- or FITC-rat anti-mouse CD45R/B220 (clone RA3–6B2), PE- or APC-hamster anti-mouse CD3\( \varepsilon \) (clone 145–2C11), PerCP-Cy5.5-mouse anti-mouse CD45.2 (clone 104), biotin- or FITC-rat anti-mouse CD44 (clone 1M7), FITC- or PE-rat anti-mouse CD8\( \varepsilon \) (clone 53–6.7), and APC-rat anti-mouse CD8a (clone H57–597) were from BD Biosciences.

Pacific Blue\( ^{\text{R}} \) and FITC-mouse anti-mouse CD45.2 (clone 104), Pacific Blue-rat anti-mouse CD44 (clone 1M7), PerCP/Cy5.5- and APC-rat anti-mouse CD127 (IL-7R\( \alpha \)) (clone SB/199), PE-mouse anti-mouse CD45.1 (clone A20) mAbs and APC-Cy7-Streptavidin\( ^{\text{TMTM}} \) were from BioLegend (San Diego, CA). APC-Cy7-streptavidin was from ebioscience (San Diego, CA); PE-rat anti-mouse CD25 (clone PC61) Ab was from Cedarlane Laboratories Ltd. (Burlington, ON, Canada).

**Generation of Bone Marrow Chimeras**—Eight- to 10-week-old C57BL/6 (CD45.2) \( \times \) C57B6.SJL (CD45.1) F1 mice were irradiated at 1,100 rads. Twenty-four h later, they received 4 \( \times \) 10\( ^6 \) T cell-depleted bone marrow cells in a 1:1 ratio from C57/B6.SJL and dKO mice of the C57BL/6 background. WT mouse bone marrow was used as control. Eight to 10 weeks after bone marrow transplantation, spleen cells of the recipients were analyzed by flow cytometry.

**Immunoblotting and Immunoprecipitation**—dKO or WT control spleen T cells and CD4 cells were purified with EasySep\( ^{\text{TMTM}} \) T Cell Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) or Miltanyi magnetic beads (Miltanyi Biotech, Bergisch Gladbach, Germany), respectively. These cells were reacted with or without IL-7 (20 ng/ml) at 37 °C for the periods indicated. The cells were then lysed in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Complete\( ^{\text{TM}} \) Protease Inhibitor Mixture and PhosSTOP Phosphatase Inhibitor Mixture, Roche Diagnostics). The lysates were resolved on 8% SDS-PAGE. Proteins on the gels were transferred to nitrocellulose membrane and blotted with rabbit anti-mouse phospho-STAT5 (Tyr-694) mAb (clone C11C5; 1:1,000 dilution; Cell Signaling Technology, Danvers, MA) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare). The membranes were then stripped and re-blotted with rabbit anti-mouse STAT5 mAb (clone 3H7; 1:1,000 dilution; Cell Signaling Technology) to assess total STAT5 expression. Signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

For immunoprecipitation, the cells were lysed and the lysates (500 \( \mu \)g/sample) were precipitated with rabbit anti-Myc polyclonal Ab-coated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 4 h. The precipitated proteins were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes, which were blotted with HRP-conjugated rat anti-HA mAb (clone 3F10, Roche Diagnostics). The membranes were then stripped and re-blotted with HRP-conjugated...
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rabbit anti-Myc Ab (Santa Cruz Biotechnology) for the detection of Myc-tagged proteins.

**Assessment of in Vivo T Cell Proliferation—**In vivo T cell proliferation was monitored with carboxyfluorescein succinimidyl ester (CFSE) labeling. T cells from dKO mice or control WT mice in the C57BL/6 background (CD45.2⁺) were labeled with CFSE (5 μM for 5 min at room temperature). Four million labeled T cells in 200 μl of PBS were transferred intravenously to sublethally irradiated (600 rads) B6.SJL mice (CD45.1⁺). Six days later, recipient spleen cells were stained with Pacific Blue-rat anti-CD45.2, PerCP-rat anti-CD4 or APC-rat anti-CD8 mAbs, and analyzed by 4-color flow cytometry.

**IL-7Rα Internalization in CD4 and EL4 Cells—**WT naïve CD4 cells from lymph nodes were first activated in wells precoated with hamster anti-mouse CD3ε mAb (clone 145–2C11, BD Biosciences) and rat anti-mouse CD28 mAb (clone 37.51.1, Cedarlane Laboratories) for 48 h to increase cell cytoplasm content for better visualization during imaging. The cells were washed and left resting for 4 days until their IL-7Rα expression returned to the pre-stimulation level. These activated CD4 and EL4 cells were cultured in medium with or without IL-7 (20 ng/ml) for 1–3 h, washed, and stained with FITC-rat anti-mouse CD4 mAb (clones GK1.5, BD Biosciences) or FITC-rat anti-mouse Thy1.2 mAb (clone 53–2.1, BD Biosciences). The cells were then fixed with 4% paraformaldehyde on ice for 30 min and permeabilized for 30 min with ice-cold PBS containing 0.3% Triton X-100. After being blocked with 0.25% gelatin, 0.01% saponin, 1% rat serum (blocking buffer), they were incubated for 1 h with biotinylated rat anti-mouse IL-7Rα (clone SB/199, BioLegend), washed in blocking buffer, and stained with streptavidin-conjugated Alexa Fluor-568 for an additional hour. Fluorescence signals were detected with a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA).

**Reverse Transcription-Quantitative Polymerase Chain Reaction—**IL-7Rα mRNA levels were measured by RT-quantitative PCR. Forward primer 5’-GGATGGAGACCTAGAGATG-3’ and reverse primer 5’-GGATTGAGCTTACCCT-3’ were used to generate a 175-bp fragment for mouse IL-7Rα. PCR conditions for the reactions were as follows: 2 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 10 s at 94 °C, 20 s at 58 °C, and 20 s at 72 °C. β-Actin mRNA levels were considered as internal controls. The data were first calculated as signal ratios of Efnb1 mRNA/β-actin mRNA and Efnb2 mRNA/β-actin mRNA. These ratios in different experiments were then normalized, and the final data were expressed as relative expression of IL-7Rα mRNA with the signal ratio of WT cells set as 1 unit.

**Efnb1 and Efnb2 Overexpression in CHO Cells—**PCR fragments containing the full-length coding sequences of Efnb1 and Efnb2 were retrieved from cDNA clones 3602195 and 6827408 from Open Biosystems (Huntsville, AL), employing the primer pairs 5’-ACCCG GCCGC ATCCT GAAGT-3’ (forward)/5’-ACCTC GAGCA TGCTG GG-3’ (reverse) and 5’-ACCCG GCCGC AGAAC TGGA GCGGC TTGGG-3’ (forward)/5’-ACCTC GAGAA GAAAC AGGTG-3’ (reverse). Fragments were first subcloned into pCR-TA Vector® (Invitrogen) and subsequently into an episomal mammalian expression vector pCEP4™ (Invitrogen) at NotI and XhoI sites downstream of the CMV promoter. The resulting plasmids were named pCEP-Efnb1 and pCEP-Efnb2. EL4 cells were transfected with these constructs by electroporation. The transfectants were selected with hygromycin B to achieve stable Efnb1 and Efnb2 overexpression. For Efnb1 and Efnb2 overexpression in CHO cells, the full-length coding sequences of Efnb1 and Efnb2 were cloned into p-ReceiverM08 (Genecopoeia, Rockville, MD), and constructs named p-Receiver-Efnb1-Myc and p-Receiver-Efnb2-Myc served for stable expression of C-terminal Myc-tagged Efnb1 and Efnb2. The stable transfectants were selected with G418, and then transiently transfected with p-ReceiverIL-7Rα-HA (Genecopoeia) for C-terminal HA-tagged IL-7Rα expression; the cells were harvested after 30 h for immunoprecipitation studies.

**Cell Preparation and FRET—**The direct association between IL-7Rα and Efnb1 or Efnb2 was assessed by FRET. Efnb1-EL4 and Efnb2-EL4 cells were first incubated on ice for 45 min with biotinylated rat anti-IL-7Rα mAb (clone SB/199, BioLegend) and goat anti-mouse Efnb1 Ab or goat anti-mouse Efnb2 Ab (both from R & D Systems), respectively. As negative controls, Efnb1-EL4 and Efnb2-EL4 cells were incubated with biotinylated rat anti-mouse Thy1.2 mAb (clone 30H12, BioLegend) and goat anti-mouse Efnb1 Ab or goat anti-mouse Efnb2 Ab, respectively. The test and control cells were reacted with Alexa Fluor-488-conjugated streptavidin for IL-7Rα (for both cross-linking and staining of IL-7Rα; Invitrogen), and with rhodamine-conjugated donkey anti-goat IgG F(ab’)2 fragments (for both cross-linking and staining of Efnb1 and Efnb2; Jackson ImmunoResearch Laboratories) at 4 °C. The cells were transferred to a 37 °C water bath and incubated for 10 min to allow cross-linking to occur; then, they were immediately fixed with 4% paraformaldehyde. Finally, the cells were washed, mounted on glass slides, and the FRET signal was examined under a Leica TCS SP5 laser-scanning confocal microscope. Alexa Fluor-488 was the donor fluorophore, and rhodamine, the acceptor fluorophore.

FRET was measured with both acceptor photobleaching (AB) and sensitized emission (SE) by FRET AB Wizard and FRET SE Wizard software (Leica Microsystems Inc.). All the necessary controls for AB and SE, such as cells with various single fluorescence staining, were performed to satisfy background deductions in calculating FRET efficiency, as required by the software.

AB FRET efficiency was calculated by fluorescence intensity of the donor before (Dpre) and after (Dpost) acceptor-selective photo bleaching, according to the following formula: AB FRET efficiency = (Dpost − Dpre)/Dpost. SE FRET efficiency was calculated based on the formula described by Woulters et al. (16): SE FRET efficiency = (B − A × B/C × C)/C; where B is FRET (indirect acceptor signal); A is donor emission (donor signal); b is the donor emission cross-talk ratio obtained from samples stained with donor fluorescence only: b = Bdonor/Adonor; C is acceptor emission (direct acceptor signal); c is the acceptor excitation cross-talk ratio obtained from samples stained with acceptor fluorescence only: c = Bandonor/Cacceptor.
RESULTS

Reduced Expression of IL-7Rα in Thymocytes and Peripheral T Cells from T Cell-specific Efnb1 and Efnb2 dKO Mice—To study the function of Efnb1 and Efnb2 in the T cell compartment, we generated conditional dKO mice with T cell-specific deletion of Efnb1 and Efnb1, using a proximal Lck promoter-driven Cre recombinase system. The proximal Lck promoter-caused Efn1 and Efnb2 deletion became effective starting from DN3 (17). Mice with floxed Efnb1/Efnb1 served as controls and were designated hereafter as wild type (WT). dKO mice manifested a significant phenotype, including about a 2-fold decrease in thymus weight and cellularity, and about a 2-fold reduction in spleen weight and spleen T cell number (18). Interestingly, we noticed that IL-7Rα expression was suppressed in most dKO thymocyte subpopulations and the decline was significant in DN3, DN4, CD4SP, and CD8 SP cells (Fig. 1A). DP cells are known to have low IL-7Rα expression (19), which could explain the less than significant diminution of this subpopulation. In the periphery, CD3+ T cells but not B cells from the control spleen expressed high IL-7Rα levels (Fig. 1B). IL-7Rα expression was significantly decreased in dKO spleen CD3+ T cells.

Because moderate T lymphopenia (18) occurred in dKO mice, it might in theory trigger a compensatory increase of IL-7 secretion, which could, in turn, down-regulate IL-7Rα in T cells. To investigate this possibility, we co-transplanted dKO or WT bone marrow cells in the CD45.2 background (C57BL/6) along with bone marrow cells from B6.SJL mice of the CD45.1 background. Cells from dKO donors competed poorly with cells from WT bone marrow cells in the CD45.2 background (C57BL/6) as a mechanism to increased IL-7 levels (19), which contributed to compromised homeostatic expansion (2). We transferred CFSE-labeled dKO or WT spleen T cells into lethally irradiated B6.SJL mice. As seen in Fig. 1E, the transferred dKO CD4 and CD8 T cells both showed significantly lower homeostatic proliferation in vivo compared with control WT T cells. Such compromised proliferation was consistent with the failed competition of dKO cells against B6.SJL cells as depicted in Fig. 1C. This suggests that reduced IL-7Rα expression in T cells does have functional consequences and contributes to compromised homeostatic expansion in vivo.

Efnb1 and Efnb2 Retard IL-7Rα Internalization in T Cells—The mechanism by which Efnb1 and Efnb2 modulate IL-7Rα expression was investigated herewith. Rapid internalization of IL-7Rα upon IL-7 engagement (8) was confirmed with both T cell lymphoma EL4 cells (Fig. 2A) and WT lymph node CD4 cells (Fig. 2B). IL-7Rα was detectable on the surface of these cells by confocal microscopy in the absence of IL-7 (Fig. 2, A and B, top row, panel I), and was located at a similar position as cell surface Thy1.2 or CD4 (Fig. 2, A and B, top row, panels II and III). After 3 h of IL-7 exposure, Thy1.2 and CD4 remained on the cell surface (Fig. 2, A and B, middle row, panel II) but IL-7Rα, being still detectable, was largely internalized and moved into the cytoplasm inside Thy1.2 or CD4 circles (Fig. 2, A and B, middle row, panels II and III). At the same time, IL-7Rα expression was drastically reduced on the surface of EL4 cells and spleen CD4 cells (Fig. 2, A and B, bottom row) upon IL-7 stimulation, whereas Th1.2 and CD4 expression on these cells remained unchanged in the absence or presence of IL-7, according to flow cytometry, further proving that IL-7-triggered IL-7Rα internalization in these cells.

We further demonstrated that after receiving IL-7 stimulation, WT naive CD4 T cells (CD44loCD62Lhi) showed a time-dependent reduction (assayed at 20, 50, and 90 min) of IL-7Rα on the cell surface (Fig. 3A, left panel), mainly due to its internalization, as described above. Efnb1 and Efnb2 deletion (i.e. dKO) in these cells resulted in a greater rate of IL-7Rα reduction (Fig. 3A, right panel), whereas the IL-7Rα mRNA level was not affected by the absence of Efnb1 and Efnb2, or the presence of IL-7 (Fig. 3B) within the 90-min period, indicating that IL-7Rα down-regulation in CD4 cells upon IL-7 stimulation occurs at the post-translational level in this period.

We wondered whether the opposite was also true: i.e. Efnb1 and Efnb2 overexpression would retard IL-7Rα down-regulation upon IL-7 stimulation. For this purpose, T cell lymphoma EL4 cells were stably transfected with Efnb1 and Efnb2 expression constructs pCEP-Efnb1 or pCEP-Efnb2, respectively. Efnb1 and Efnb2 overexpression on the cells was confirmed by flow cytometry (supplementary Fig. S1A), and the cells were named Efnb1-EL4 and Efnb2-EL4, respectively. Their cell surface IL-7Rα down-regulation upon IL-7 stimulation was significantly retarded, when compared with EL4 cells transfected with empty vector (vector-EL4; Fig. 3C). On the other hand, IL-7Rα mRNA levels in Efnb1-EL4 and Efnb2-EL4 cells were comparable with vector-EL4 cells, in the absence or presence of IL-7 (Fig. 3D), confirming that IL-7Rα down-regulation is at the post-transcriptional level during this time frame. Taken together, these findings confirm that the presence of Efnb1 and Efnb2 retards IL-7Rα down-regulation on the T cell surface upon IL-7 stimulation; conversely, the absence of Efnb1 and Efnb2 accelerates IL-7-triggered IL-7Rα down-regulation. In contrast to IL-7Rα, Efnb1 and Efnb2 expression after anti-Efnb1 or -Efnb2 Ab cross-linking (supplemental Figs. S1B and S1C, middle row) or IL-7 treatment (bottom row) remained largely unchanged, compared with cells cultured in plain medium (upper row).

Efnb1 and Efnb2 Physically Interact with IL-7Rα and Anchor It on the Cell Surface—We then questioned whether Efnb1 and Efnb2 associated physically with IL-7Rα as a mechanism to
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A

Relative cell number

DN

17.9±3.9% 14.5±3.2%
P<0.05

Isotypic control

WT

dKO

DN2

41.3±4.8%

DN3

12.6±1.1%

DN4

16.1±1.5%

P<0.05

P>0.05

P<0.05

P<0.01

DP

12.4±0.8%

37.0±3.8%

9.1±1.6%

6.4±1.6%

P<0.05

P<0.05

P<0.05

P<0.05

CD4SP

38.5±10.4%

29.6±5.4%

P<0.05

CD8SP

38.7±5.0%

26.0±3.9%

P<0.01

B

Relative cell number

B220+ cells

5.7±3.5%

-7.1±3.9%

P>0.05

CD3+ cells

46.1±7.5%

32.2±13.3%

P<0.05

C

Relative cell number

CD4+ cells

40.0±6.6%

40.1±3.6%

P<0.05

CD8+ cells

47.0±10.3%

46.7±11.9%

P>0.05

D

Spleen T cells

Spleen CD4 T cells

IL-7

0 min

0 20 0 20

p-STAT5

dKO

WT

STAT5

dKO

WT

E

Relative cell number

CD4+ cells

WT

dKO

CD8+ cells

WT

dKO

CFSE

44.9±4.6%

20.9±2.5%

P<0.01

75.2±4.0%

37.0±2.7%

P<0.001
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**FIGURE 1. Reduced IL-7Rα expression in dKO thymocytes and T cells.** All experiments in this figure were repeated 3 times or more, and representative results are reported. Flow cytometry histograms show the mean ± S.D. of percentages of IL-7Rα-positive cells (A–C) and the percentages of cells with reduced CFSE (E) from all repetitions. p values (Student’s t test) are indicated. A, IL-7Rα expression in DN, DN2, DN3, DN4, DP, CD4SP, and CD8SP thymocytes. Thymocytes were gated on DN, DN2, DN3, DN4, DP, CD4SP, or CD8SP cells, and their IL-7Rα expression was analyzed by flow cytometry. B, IL-7Rα expression in T and B cells. Spleen cells were gated on B220- B or CD3+ T cells, and their IL-7Rα expression was analyzed by flow cytometry. C, reduced IL-7Rα expression in spleen CD4 and CD8 T cells derived from dKO bone marrow cells in irradiated recipients. T cell-depleted dKO (lower row) and WT (upper row) bone marrow cells (CD45.1+) were mixed with T cell-depleted bone marrow cells from B6.SJL competitors (CD45.1-) at 1:1 ratio and transplanted to lethally irradiated C57BL/6 x B6.SJL F1 recipients. After 60 days, spleen CD4 and CD8 cells were analyzed by flow cytometry. CD4+ T cells were derived from dKO or WT bone marrow, CD45.1+ cells were from competing B6.SJL bone marrow cells, and CD45.1+/CD45.2- cells were from residual cells of the recipients. CD4 and CD8 cells from CD45.2+ or CD45.1+ spleen cells were gated and analyzed for IL-7Rα expression. D, reduced STAT5 phosphorylation in dKO T cells upon IL-7 stimulation. dKO and WT spleen T or CD4 cells were stimulated with IL-7 (10 ng/ml) at 37 °C for the periods indicated, and then lysed. Phosphorylated STAT5 (p-STAT5) in lysates was assessed by immunoblotting. Total STAT5 protein in lysates was evaluated by re-blotting the same membrane with anti-STAT5 Ab. E, the presented dKO T cells failed homeostatic expansion in sublethally irradiated recipients. B6.SJL mice (CD45.1+) were sublethally irradiated at 600 rads and transplanted intravenously with 4 × 10⁶ CFSE-labeled spleen cells from dKO or WT mice (both CD45.2+). The histograms represent profiles of CFSE-positive cells gated on CD4+CD45.2+ and CD8+CD45.2+ cells.

**FIGURE 2. IL-7Rα internalization in EL-4 cells and spleen CD4 T cells.** EL4 cells (A) were cultured in medium without IL-7 (top row) or with IL-7 (20 ng/ml, middle row) for 3 h, washed, and stained with FITC-anti-Thy1.2 mAb. WT naive CD4 cells (B) were from lymph nodes were first activated with soluble anti-CD3ε and anti-CD28 mAb for 48 h to increase cell cytoplasm content for better visualization during imaging. The cells were left to rest for 4 days. These activated cells were cultured in medium without IL-7 (top row) or with IL-7 (20 ng/ml, middle row) for 3 h, washed, and stained with FITC-anti-CD4 mAb. The cells were then fixed, permeabilized, and stained with biotinylated anti-IL-7Rα, followed by Alexa Fluor-568-conjugated streptavidin. Fluorescent signals were detected with confocal microscopy. Merged images (panel III) show the position of IL-7Rα (in red) relative to cell surface Thy1.2 (in green) or CD4 (in green). The experiments were repeated more than 3 times and representative data are reported. The cells were also stained for IL-7Rα plus Thy1.2 or CD4 without permeabilization and analyzed by flow cytometry (bottom row). Solid line, cells cultured in medium; dotted line, cells cultured in the presence of IL-7. The mean ± S.D. of fluorescence intensity in more than 3 experiments are indicated in the histograms, and p values are presented (Student’s t test).

reduce IL-7Rα internalization. When Efnb1-EL4 cells were cross-linked with anti-Efnb1 Ab plus anti-IL-7Rα Ab, both Efnb1 and IL-7Rα formed caps within 10 min before IL-7Rα internalization occurred. Efnb1 and IL-7Rα co-localized in the cap on the cell surface (Fig. 4A, left panel), whereas Efnb1 and Thy1.2 did not co-cap when Efnb1-EL4 cells were cross-linked with anti-Efnb1 Ab plus anti-Thy1.2 Ab (Fig. 4A, right panel). Similarly, Efnb2 and IL-7Rα co-capping was also manifested in Efnb2-EL4 cells when cross-linked with anti-Efnb2 Ab plus anti-IL-7Rα Ab (Fig. 4B, left panel), but Efnb2 and Thy1.2 did not co-cap when Efnb2-EL4 cells were cross-linked with anti-Efnb2 plus anti-Thy1.2 Ab (Fig. 4B, right panel). The co-migration of Efnb1 or Efnb2 with IL-7Rα raises the possibility that Efnb1 and Efnb2 may directly associate with IL-7Rα on the T cell surface.

It should be noted that if only 1 Ab (i.e. anti-IL-7Rα or anti-Efnb1 Ab) was used in the cross-linking step, IL-7Rα and Efnb1 (or Efnb2) would still undergo discernible but faint co-capping between Efnb1 and IL-7Rα, or between Efnb2 and IL-7Rα, respectively, under the microscope, but the resulting images were not satisfactory for illustration. This was mainly due to a technical difficulty: the cells needed to be fixed to terminate
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cross-linking, but the other Ab (the one not deployed in initial cross-linking) could not stain fixed cells as well as fresh cells, whereas in double cross-linking experiments (Fig. 4), both Abs (i.e., anti-IL-7Ra and anti-Efnb1 Ab) reacted with fresh cells.

Two methods of fluorescence resonance energy transfer (FRET), i.e., AB and SE, were employed to assess such a possibility. Fig. 5, A and B, show FRET from IL-7Ra to Efnb1 and IL-7Ra to Efnb2, respectively, using AB. In both figures, panel I presents micrographs illustrating immunofluorescence staining of IL-7Ra versus Efnb1 in Efnb1-EL4 cells, and IL-7Ra versus Efnb2 in Efnb2-EL4 cells. The upper half of the figures illustrates Efnb1-EL4 cells or Efnb2-EL4 cells without cross-linking of their IL-7Ra or Efnbs. Efnb1, Efnb2, and IL-7Ra were distributed rather uniformly on the cell surface before AB. After AB, acceptor (Efnb1 or Efnb2) but not donor (IL-7Ra) signals were drastically weakened, proving the effectiveness of acceptor-specific bleaching. The lower half of the figures show Efnb1-EL4 or Efnb2-EL4 cells cross-linked with anti-Efnb1/anti-IL-7Ra Abs or anti-Efnb2/anti-IL-7Ra Abs, respectively. In these cells, Efnb1 or Efnb2 were co-capped with IL-7Ra; AB depleted Efnb1 and Efnb2 but not IL-7Ra signals. The donor fluorescence intensity of selected areas (in colored circles) in the bleached region (rectangles) and control areas outside the bleached region of sample cells (Fig. 5, A and B, panels II) was quantified, and data from these representative cells are listed in the tables in panel III of Fig. 5, A and B. In both Efnb1-EL4 cells (Fig. 5A and table in panel III) and Efnb2-EL4 cells (Fig. 5B, table in panel III), without (upper half of the tables) or with cross-linking (lower half of the tables), there was a bigger increase in donor (IL-7Ra) fluorescence intensity in the area inside the AB region, compared with the area outside the bleached region. The mean ± S.D. of FRET efficiency (from IL-7Ra to Efnb1 or IL-7Ra to Efnb2) of areas inside and outside the bleached regions of more than 10 randomly selected cells are shown in Fig. 5, A and B, panel IV. The results indicate that IL-7Ra associates with Efnb1 and Efnb2 constitutively and after IL-7Ra engagement. The results based on AB FRET were verified with SE FRET. As illustrated in Fig. 6A, FRET efficiency based on energy transfer from IL-7Ra to Efnb1 or IL-7Ra to Efnb2 was significantly higher than that of the controls (i.e., FRET efficiency from Thy1.2 to Efnb1 or from Thy1.2 to Efnb2), whether IL-7Ra/Efnb1 or IL-7Ra/Efnb2 was cross-linked (Fig. 6A, right panel) or not (Fig. 6A, left panel). It should be noted that SE FRET efficiency in both controls was near-null.

Immunoprecipitation was performed to further prove the physical interaction between Efnb1/Efnb2 and IL-7Ra. CHO cells were first stably transfected with Myc-tagged Efnb1 or Efnb2, and then transiently transfected with HA-tagged IL-7Ra. The overexpression of Efnb1, Efnb2 (supplemental Fig. S2A), and IL-7Ra (supplemental Fig. S2B) in these cells was confirmed by flow cytometry. In anti-Myc immunoprecipitation of CHO cells with Efnb1-Myc/IL-7Ra-HA double transfection or Efnb2-Myc/IL-7Ra-HA double transfection (Fig. 6B, first and last lanes), IL-7Ra-HA could be detected by anti-HA Ab in immunoblotting. No HA signals were detected in CHO cells with IL-7Ra-HA single transfection (Fig. 6B, middle lane), excluding the possible carry over of IL-7Ra-HA by anti-Myc Ab-conjugated beads. The presence of Efnb1-Myc and Efnb2-Myc in the first and last lanes was confirmed by anti-Myc Ab-immunoblotting (Fig. 6B, lower panel). With FRET and immunoprecipitation, we thus demonstrated that IL-7Ra directly associates with Efnb1 and Efnb2.

**DISCUSSION**

In the present study, we discovered that Efnb1 and Efnb2 physically interact with IL-7Ra, and modulate its internalization and signaling, with functional consequences. IL-7Ra expression is controlled upon its exposure to IL-7, and such regulation occurs at several levels. Longer term (several hours to several days) IL-7 exposure leads to down-regulation of IL-7Ra at the mRNA level (8). In human CD8 cells, it is reported that a high IL-7 concentration results in increased shedding of IL-7Ra into culture supernatants (20), but the process takes 24 h. Immediate IL-7Ra expression is regulated by internalization, which transpires within 20–180 min after it encounters IL-7. This process presumably serves as a negative regulatory loop to control the strength and duration of IL-7R signaling (21).

In contrast to IL-7Ra, Efnb1 and Efnb2 expression levels on the cell surface are not subjected to modulation by IL-7 engagement, nor are these 2 molecules internalized after being engaged by their agonists. The association between Efnb1/Efnb2 and IL-7Ra thus enables the former to act as anchors to moor IL-7Ra on the cell surface and prevent it from rapid internalization. In the absence of Efnb1 and Efnb2, this mooring effect is lost. Ex vivo examination of thymocytes and peripheral T cells disclosed that IL-7Ra levels on these cell surfaces are reduced, likely due to faster internalization caused by ambient or stimulated IL-7 in vivo. It should be noted that the mooring
Effect of Efnb1 and Efnb2 is additive, as the reduction of IL-7Rα in thymocytes and T cells only becomes apparent when both, but not only one of them, are deleted.

The effect of Efnb1 and Efnb2 on IL-7-stimulated IL-7Rα down-regulation on the cell surface seems to have certain specificity. IL-7Rα on T cells is also down-regulated after TCR stimulation (22), and this is not an IL-7-dependent event. We showed that such down-regulation occurred at a slower pace compared with IL-7-induced down-regulation, only being apparent 16 h after TCR stimulation and not influenced by a lack of Efnb1 and Efnb2 (supplemental Fig. S3A, left panel). An explanation is that such down-regulation is mainly at the transcription level (supplemental Fig. S3B, right panel), which is not subjected to the Efnb1 and Efnb2 anchoring effect on the cell surface. The nonpromiscuous nature of the Efnb1/Efnb2 effect was also demonstrated in the case of IL-6Rα. IL-6Rα on T cells are also internalized after encountering IL-6 (23), but this event was not affected by Efnb1 and Efnb2 deletion.

**FIGURE 4.** IL-7Rα co-localizes with Efnb1 and Efnb2 after IL-7Rα/Efnb1 or IL-7Rα/Efnb2 cross-linking. Efnb1-EL4 cells (A) and Efnb2-EL4 cells (B) were cultured in plain medium (without cross-linking), or cross-linked with anti-Efnb1 plus anti-IL-7Rα Ab, anti-Efnb2 plus anti-IL-7Rα Ab, anti-Efnb1 plus anti-Thy1.2 Ab, or anti-Efnb2 anti-thy1.2, as indicated. IL-7Rα, Thy1.2, Efnb1, and Efnb2 expression on the cell surface was detected by confocal microscopy.
**FIGURE 5. Efnb1/Efnb2 associate with IL-7Rα according to AB FRET.** Efnb1-EL4 (A) and Efnb2-EL4 cells (B) were analyzed for interaction between Efnb1 and IL-7Rα, and between Efnb2 and IL-7Rα, respectively, by AB FRET. The cells were not Ab-cross-linked (upper half of the figures) or were Ab-cross-linked (anti-IL-7Rα Ab plus anti-Efnb1 Ab in A, and anti-IL-7Rα Ab plus anti-Efnb2 Ab in B; lower half of the figures). Panel I of A and B shows IL-7Rα (donor fluorophore in AB FRET) expression and Efnb expression (acceptor fluorophore in AB FRET) before and after AB. Panel II of A and B illustrates the AB region (in rectangle) and selected areas (in colored circles) of a sample cell for AB FRET analysis. Panel III of A and B presents tables containing fluorescence intensity of a bleached area (white circle) and unbleached area (red circle) in the sample cell of panel II. Panel IV presents bar graphs summarizing AB FRET efficiency based on data from more than 10 randomly selected cells (2 circles per cell inside the bleached area and 2 circles per cell outside the bleached area).
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**FIGURE 6. Interaction between Efnb1/Efnb2 and IL-7Rα according to SE FRET and immunoprecipitation.** A, SE FRET. Efnb1-EL4 cells and Efnb2-EL4 cells were cross-linked with a pair of Abs (anti-IL-7Rα or anti-Thy1.2 Ab plus anti-Efnb1 Ab for Efnb1-EL4 cells, and anti-IL-7Rα or anti-Thy1.2 Ab plus anti-Efnb2 Ab for Efnb2-EL4 cells), or remained untreated, as indicated. Mean ± S.D. of SE FRET efficiency between IL-7Rα and Efnb1, between IL-7Rα and Efnb2, between Thy1.2 and Efnb1, and between Thy1.2 and Efnb2 are presented. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively (Student's $t$ test). B, immunoprecipitation. CHO cells stably expressing Myc-tagged Efnb1 or Efnb2 were transiently transfected for HA-tagged IL-7Rα expression. The cell lysates were precipitated with anti-Myc Ab-coated agarose beads. The proteins were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes, which were sequentially blotted with anti-HA Ab and anti-Myc Ab. IP, immunoprecipitation; WB, Western blotting. Efnb1-Myc + IL-7Rα-HA, CHO cells stably expressing Myc-tagged Efnb1 were transiently transfected for HA-tagged IL-7Rα expression; Efnb2-Myc + IL-7Rα-HA, CHO cells stably expressing Myc-tagged Efnb2 were transiently transfected for HA-tagged IL-7Rα expression; IL-7Rα-HA, WT CHO cells were transiently transfected for IL-7Rα expression as negative control for immunoprecipitation. All experiments in this figure were repeated twice or more, and representative cells or data are reported.

(supplemental Fig. S3B). With that said, it is still possible that Efnb1 and Efnb2 anchor cell surface receptors other than IL-7Rα and modulate their internalization.

The anchoring effect of Efnb1 and Efnb2 on IL-7Rα was not affected by Efnb1 and Efnb2 engagement, as the rate of IL-7Rα internalization upon IL-7 stimulation in Efnb1-EL4 and Efnb2-EL4 cells was not influenced by solid phase anti-Efnb1 and anti-Efnb2 Ab in culture wells (supplemental Fig. S4, bottom row), when compared with those cultured in uncoated wells (supplemental Fig. S4, top row).

In the absence of Efnb1 and Efnb2, IL-7Rα expression was down-regulated, likely as a consequence of enhanced internalization. What is the functional outcome of such increased IL-7Rα down-regulation? A recent report suggests that IL-7Rα internalization is necessary for IL-7R signaling, because treating leukemic cells with hyperosmotic sucrose, which inhibits IL-7Rα internalization, results in reduced JAK1/3, STAT5, and AKT signaling (8). It follows that increased IL-7Rα internalization that causes decreased IL-7Rα expression should promote IL-7R signaling. The validity of such a conclusion is debatable, primarily because hyperosmotic treatment disturbs the entire cell biology, including multiple signaling pathways. A more intuitive conclusion is that reduced IL-7Rα expression in the T cell compartment evoked by increased internalization will lead to compromised IL-7-dependent thymocyte and T cell functions. Several functional studies support such a conclusion. We presented evidence that STAT5 phosphorylation, a critical IL-7R signaling event, was abated in dKO CD4 cells after IL-7 stimulation. As shown in Fig. 1D, dKO CD4 and CD8 cells manifested compromised in vivo homeostatic proliferation, an IL-7-dependent event. Therefore, our data indicate that one of the physiological functions of Efnb1 and Efnb2 is to stabilize IL-7Rα expression and enhance IL-7 signaling in the T cell compartment. Under physiological conditions, the modulation of such stabilization probably depends on Efnb1 and Efnb2 expression levels.

The finding that Efns directly associate with other receptors and influence their stability at the cell surface reveals a previously unknown modus operandi of Efns. It is conceivable that Efns other than Efnb1 and Efnb2 might also have such a capability, and they might affect not only IL-7Rα stability and function but also other receptors or cell surface molecules. In a broader sense, such effects could be viewed as part of Efns reverse signaling, as this imparts changes of signaling into the cells via Efns, although such reverse signaling does not necessarily need to be triggered by Ephs, and is not necessarily transmitted directly via Efns, but via associated receptors. Recently, Sawamiphak et al. (24) and Wang et al. (25) reported that, in endothelial cells, Efnb2 facilitates VEGFR2 and VEGFR3 internalization, which, for VEGFRs, is an essential step for their signaling. Wang et al. (25) observed rapid cell surface co-localization of Efnb2 and VEGFR3 on cells upon VEGF-C stimulation. Also, Efnb2 physically binds to VEGFRs according to immunoprecipitation (25), an additional example of Efnb modulating the function of other receptors at the post-translational level. It seems that whether such interaction retards or enhances the internalization of other molecules is case-dependent; factors that determine whether internalization is reduced or enhanced remain to be elucidated.

Most documented functions of Ephs and Efns are related to pattern formation, and depend on interaction between Ephs and Efns on neighboring cells. The findings of our current study on the effect of Efns on post-translational modulation of IL-7Rα in the T cell compartment have revealed Efns functions have little to do with pattern formation. The mooring effect of Efns on IL-7Rα does not seem to require direct input from Ephs, although it is conceivable that factors modulating Efnb1 and Efnb2 expression will in turn alter IL-7Rα expression. Such a novel modus operandi of Efnb1 and Efnb2 in regulating the functions of other cell surface receptors at the post-translational level has revealed a previously underappreciated regulatory role between different cell surface receptors.

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