Mouse EphrinB3 Augments T-cell Signaling and Responses to T-cell Receptor Ligation*

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Ephrins (EFN) are cell-surface ligands of Ephs, the largest family of cell-surface receptor tyrosine kinases. The function of EFNs in the immune system has not been well studied, although some EFNs and Ephs are expressed at high levels on certain leukocytes. We report here that EphrinB3 and its receptors (collectively called EFNB3Rs, as EFNB3 binds to multiple EphBs) were expressed in peripheral T cells and monocytes/macrophages, with T cells being the dominant EFNB3+ and EFNB3R+ cell type. Solid-phase EphrinB3-Fc in the presence of suboptimal anti-CD3 crosslinking enhanced T-cell responses in terms of proliferation, activation marker expression, interferon-γ but not interleukin-2 production, and cytotoxic T-cell activity. EFNB3R costimulation in the presence of phorbol 12-myristate 13-acetate was insensitive to cyclosporin A, similar to CD28 costimulation, suggesting they share a part of the signaling pathway. After crosslinking, T-cell receptor and EFNB3R congregated into aggregated rafts, and this provided a morphological basis for signaling pathways of T-cell receptor and EFNB3R to interact. Solid-phase EphrinB3-Fc augmented p38 and p44/42 MAPK activation further downstream of the signaling pathway. These data suggest that EphrinB3 is important in T-cell/T-cell and T-cell/antigen-presenting cell collaboration to enhance T-cell activation and function.

Full T-cell activation requires costimulation in addition to T-cell receptor (TCR) ligation. Costimulation can be conferred by cell-surface molecules. For example, the well studied B7.1 and B7.2 are two prototype costimulatory molecules expressed on antigen-presenting cells (1). Additional members of the B7 family with costimulatory function have been documented recently (2). Certain cell-surface adhesion molecules (3) and some membrane-bound tumor necrosis factor family members (4, 5) are capable of costimulation as well. In general, the costimulation increases the affinity and prolongs the interaction between T cells and antigen-presenting cells; it also allows the integration of costimulation-triggered signaling pathways with that of TCR. As a result, the T-cell response threshold to Ag is reduced, and the response to Ag is enhanced.

Ephrins (EFN) are cell-surface ligands of Eph receptor tyrosine kinases. EFNs are classified into A and B subfamilies; the former consists of six glycosylphosphatidylinositol-anchored membrane proteins (EFNA1–6), and the latter consists of three transmembrane proteins (EFNB1–3) (6). EFNs are capable of reverse transmission of signal into cells, although they are ligands (7). EFNAs bind to EphAs with loose specificity, and, likewise, EFNBs bind to EphBs (8), with the exception of EphA4, which binds both EFNA and EFNBs (9). Because both EFNs and Ephs are cell-surface proteins, they interact with each other in proximity and control cell patterning as well as directional growth. Such functions are well demonstrated in the central nervous system (10), during angiogenesis (11), and as reported recently (12, 13), in intestinal development and remodeling.

Some members of the Eph and EFN families are expressed in lymphoid organs and on some leukocytes (14–29), but we are really in the very beginning of our understanding of the function of this largest family of receptor tyrosine kinases and their ligands in immune regulation. We have reported previously that EphB6 crosslinking by mAb leads to signal transduction and apoptosis of Jurkat T cells (26) as well as to augmentation of normal human T-cell responses (20). Preliminary examination of EphB6−/− mice shows no gross anomaly in the thymic structure and thymocyte populations (21), suggesting compensatory mechanisms at work. In this study, we investigated the expression of EFNB3 and its receptors in immune cells and explored its function in regulating T-cell activity.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—A 522-bp cDNA fragment of mouse EFNB3 cDNA from positions 160 to 681 (accession number AF025288) was fetched with PCR from a mouse embryonic tissue cDNA library and cloned into pGEM-4Z (Invitrogen). The resulting construct, pGEM-4Z-mB3, served to transcribe antisense probes with SP6 RNA polymerase or to transcribe sense probes with T7 RNA polymerase using digoxigenin RNA labeling kits (Roche Diagnostics, Laval, Quebec, Canada). In situ hybridization was carried out according to instructions from the kit manufacturer.

Generation of Mouse EphrinB3-Fc—The coding sequence of the extracellular domains of mouse EFNB3 from positions 34 to 717 was cloned in-frame upstream of the human IgG1-Fc coding sequence in expression vector pCMVhFc. The constructs and pcDNA3 were then transfected...
into Chinese hamster ovary (CHO) cells with LipofectAMINE. The cells were cultured in selection medium (α-minimum Eagle's medium without ribonucleosides and deoxyribonucleosides containing 5% dialyzed fetal calf serum, 0.01 mM methotrexate, 0.8 mg/ml G418, and 0.1 mg/ml gentamycin). Stably transfected clones were handpicked after 2 weeks of culture. Fusion proteins were isolated from supernatants by protein A columns and verified by N-terminal peptide sequencing (Sheldon Biotechnology Center, McGill University, Montreal, Quebec, Canada).

**Lymphocyte Preparation and Culture**—Cells were flushed out from the BALB/c mouse spleen, and red blood cells were lysed with 0.84% NH4Cl, as described elsewhere (30). The resulting cells were referred to as spleen cells. Spleen T cells were purified by deleting mouse IgG (H+L)-positive cells from spleen cells with T-cell columns according to the manufacturer’s instructions (Cedarlane, Hornby, Ontario, Canada). In some experiments, CD4 and CD8 cells were positive-selected from the spleen cells by magnetic beads (Miltenyi Biotec, Auburn, CA). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, l-glutamine, and penicillin-streptomycin. Solid-phase EFNB3 and anti-CD3 were prepared by coating 96-well Costar 3595 plates overnight with anti-mouse CD3 (clone 2C11) in phosphate-buffered saline (PBS) at 4 °C, followed by incubating EFNB3-Fc or, as a control, normal human IgG (NH1gG) (Southern Biotechnology, Birmingham, AL) of different concentrations at 37 °C for 2 h, followed by an additional 2-h incubation on ice. In some experiments, CD4 and CD8 cells were positive-selected from the spleen cells by magnetic beads (Miltenyi Biotec). For mixed lymphocyte reaction (MLR), spleen cells from BALB/c mice were treated with mitomycin C and mixed with C57Bl/6 spleen cells at a 1:1 ratio (8 × 10^6 cells at 200 μl/well). The cells were cultured in the presence of NH1gG or Ephs (Eph A4, EphB3, and EphB4; R&D Research, Minneapolis, MN) for 5 days before being harvested.

**[3H]Thymidine Uptake Assay**—Lymphocytes were cultured in 96-well Costar 3595 plates coated with different mAbs, recombinant proteins, or reagents in solution. The cells were pulsed with [3H]thymidine for the last 16 h of culture, and [3H]thymidine uptake was measured, as described previously (31).

**Flow Cytometry**—Flow cytometry was employed for the measurement of EFNB3 expression as well as EFNB3R expression in T-cell populations. BALB/c spleen cells were stained with goat anti-EFNB3 (R&D Systems) followed by donkey anti-goat IgG-PE (Cedarlane) or with biotinylated EFNB3-Fc followed by streptavidin-PE. Anti-Thy1.2-FITC (Caltag Laboratories, Burlingame, CA) was used for the second color. To measure the expression of activation markers on T cells, spleen T cells were stained with anti-Thy1.2-PE in combination with anti-CD25-FITC (clone M-A251), anti-CD44-FITC (clone IM7), anti-CD54-FITC (clone HA58), or anti-CD69-FITC (clone FN50). All of these mAbs were obtained from PharMingen, unless indicated otherwise.

**Cytotoxic T Lymphocyte (CTL) Assay**—The assay was performed as detailed earlier (32) using transgenic 2C mouse spleen cells (in H-2 b background) were used as their T cells specific to Ld, 0.4 g protein/10^6 cells) on 51Cr-labeled P815 cells (H-2 d) as targets. This assay was performed at 37 °C for different time periods; this was followed by immediate fixation with 3% formalin. For TCR and EFNB3R staining, the cells were reacted with streptavidin-Alexa Fluor 594 (1 μg/sample) and goat anti-human IgG-Alexa Fluor 488 (1 μg/10^6 cells) on ice for 30 min. For raft and EFNB3R staining, the procedure was similar to that described above, but cholera toxin-Alexa Fluor 594 (0.5 μg/sample) was used instead of streptavidin-Alexa Fluor 594. The slides were examined under a confocal microscope.

**Immunoblotting**—Twelve-well plates were coated overnight with anti-CD3 (0.5 μg/ml, 500 μl/well) at 4 °C. After washing, the wells were incubated with EFNB3-Fc or NH1gG, both at 10 μg/ml and 500 μl/well at 37 °C for 1 to 2 h and then at 0 °C for another 2 h. BALB/c spleen T cells were seeded in the pre-coated plates at 5 × 10^5 cells/well, and the plates were centrifuged at 228 g for 5 min to achieve rapid contact between the cells and the bottom of the culture wells. The cells were then cultured at 37 °C for 2 h before being harvested. The remainder of the procedure is detailed in our previous publication (30). Briefly, the harvested cells were washed and lysed in lysis buffer for 10 min; the cleared lysates were resolved in 10% SDS-PAGE with 50 μg protein/lane and were then blotted onto polyvinylidene difluoride membranes. The membranes were subsequently hybridized with the Abs used in immunoblotting were obtained from New England Biolab (Mississauga, Ontario, Canada). Signals were revealed by enhanced chemiluminescence.

**RESULTS**

**EFNB3 and EFNB3R Expression in Leukocytes**—EFNB3 expression at the mRNA level in the spleen was examined by in situ hybridization with the liver serving as a negative control (Fig. 1). In the spleen, the EFNB3 signal was detected in the white pulp, suggesting that lymphocytes express EFNB3.

The expression of EFNB3 and EFNB3Rs on resting and activated Thy1^+^ T cells was investigated with flow cytometry by anti-EFNB3/anti-Thy1 or EFNB3-Fc/anti-Thy-1 two-color staining (Fig. 2A). EFNB3 was detectable in 18.8% resting T cells (cultured in medium), and 24-h activation of T cells with solid-phase anti-CD3 did not significantly change the level of EFNB3 expression (24.0%). EFNB3R was expressed on 30.4% resting T cells; no apparent modulation of expression (30.1%) 24 h after T-cell activation was observed. The expression of EFNB3 and EFNB3R in resting CD4 and CD8 cells (cultured in medium for 24 h) was determined by anti-EFNB3 or EFNB3-Fc one-color staining of magnetic bead-purified CD4 and CD8 cells (Fig. 2C). The expression of EFNB3 on CD4 and CD8 cells was similar (26.1 and 22.1%, respectively), although CD4 cells expressed more EFNB3Rs than CD8 cells (45.4 and 21.3%, respectively).

EFNB3 and EFNB3R were barely detectable in B cells (5.7 and 3.2%, respectively) but were expressed in a significant percentage of monocytes/macrophages (20.4 and 35.5%, respectively) (Fig. 2B).

**E. EFNB3 Enhances T-cell Proliferation and Modulates Activation Marker Expression**—Because EFNB3R is expressed in a significant portion of T cells, it is likely that EFNB3 plays a role in modulating T-cell function. Solid-phase EFNB3, in the presence of solid-phase anti-CD3, was used to assess this possibility. EFNB3-Fc, but not a control protein (normal human IgG), dose-dependently induced T-cell proliferation in the presence of a suboptimal concentration of anti-CD3 (Fig. 3A). When T cells were stimulated with an optimal amount of EFNB3-Fc and

![Image](http://www.jbc.org/)
EFNB3 Enhances Lymphokine Production and CTL Activity—We next examined the effect of EFNB3 on T-cell effector functions, such as lymphokine production and CTL activity. Again, T cells were stimulated with solid-phase anti-CD3 alone (at a suboptimal amount) or in combination with EFNB3-Fc or anti-CD28 (both at optimal amounts and on solid-phase). NHIgG was used as a negative control for EFNB3-Fc. Three representative lymphokines, IL-2, IL-4, and IFN-γ, were measured from day 1 to day 3 after stimulation (Fig. 5A). Anti-CD3 with or without NHIgG (i.e., PBS) did not trigger lymphokine production. Anti-CD28 costimulation drastically induced IL-2, IL-4, and IFN-γ, as expected. EFNB3-Fc led IFN-γ production

various amounts of anti-CD3, they proliferated dose-dependently in response to the latter (Fig. 3B). We also compared the costimulation by EFNB3-Fc with that by anti-CD28 mAb (Fig. 3C). Costimulation mediated by EFNB3Rs was at a moderately lower level than that mediated by the classical costimulatory molecule CD28. Anti-CD3 (at a suboptimal concentration) or EFNB3-Fc (at an optimal concentration) alone caused low level or no T-cell proliferation, respectively (last two columns of Fig. 3C). The response of CD4 and CD8 cells to EFNB3 costimulation was similar (Fig. 3D).

Interestingly, the costimulation via EFNB3Rs was insensitive to cyclosporin A (CyA). As shown in Fig. 3E, CyA, which inhibits Ca²⁺ flux-dependent T-cell activation (33, 34), could effectively suppress ionomycin and PMA-induced T-cell proliferation but failed to do so for T cells activated by PMA plus solid-phase anti-CD28 or PMA plus solid-phase EFNB3. This finding suggests that CD28 and EFNB3Rs costimulation both utilize the Ca²⁺-independent pathway. Next, we assessed whether the EFNB3Rs can augment anti-CD28 costimulation in the presence of anti-CD3. At optimal CD28 concentration (10 μg/ml for coating), solid-phase EFNB3 had no additional effect (Fig. 3F). However, at suboptimal anti-CD28 concentration (5 or 2.5 μg/ml), EFNB3 could moderately enhance the costimulation. This finding suggests again that CD28 and EFNB3Rs do not employ two totally separate pathways.

EFNB3 is known to interact with three Eph receptors, i.e. EphA4, EphB3, and EphB4 (35–37). We investigated which of these mediated EFNB3-triggered costimulation. Soluble EphA4, EphB3, or EphB4 alone (3 μg/ml) or in combination were added to MLR, and thymidine uptake was measured 5 days later. As shown in Fig. 3G, EphA4 and EphB4, but not EphB3, was able to suppress MLR, suggesting the possible involvement of the former two in the EFNB3-triggered costimulation.

The expression of certain activation markers on T cells after EFNB3 costimulation was then assessed. T cells were stimulated with solid-phase anti-CD3 alone (at a suboptimal amount) or solid-phase anti-CD3 plus EFNB3-Fc (at an optimal amount). As shown in Fig. 4, anti-CD3 alone (shaded area) was unable to up-regulate the activation markers examined, i.e. CD25, CD44, CD54, and CD69. After combined stimulation with anti-CD3 and EFNB3-Fc, CD69 was significantly up-regulated, although CD25 and CD44 were not. In contrast, all of these markers were drastically up-regulated when T cells were costimulated by solid-phase anti-CD28.

These results indicate that EFNB3Rs can enhance T-cell activation to TCR stimulation, but such enhancement seems qualitatively different from that of CD28.
FIG. 3. Solid-phase EFNB3-Fc enhances T-cell proliferation upon TCR stimulation. Normal human IgG (NHlgG) served as a control for EFNB3-Fc. BALB/c T cells were cultured in wells coated with a suboptimal amount of anti-CD3 (0.8 μg/ml) and different amounts of EFNB3-Fc (A), a fixed optimal amount of EFNB3-Fc (10 μg/ml) and different amounts of anti-CD3 (B), or a suboptimal amount of anti-CD3 (0.8 μg/ml) along with optimal amounts of anti-CD28 or EFNB3-Fc (both at 10 μg/ml) (C). D, magnetic bead-purified spleen CD4 or CD8 cells were cultured in wells...
to a level comparable with but moderately lower than that caused by anti-CD28 costimulation but did not stimulate IL-4 and IL-2 production. The lack of IL-2 and IL-4 production after EFNB3 costimulation was not caused by a shift of secretion kinetics, because no production of IL-2 or IL-4 was observed at any time between days 1 and 3 after culture. This result demonstrates that EFNB3 and anti-CD28 costimulation have qualitative differences.

When mixed lymphocyte reaction was elicited in EFNB3-Fc-coated wells, CTL development was greatly enhanced (Fig. 5B), whereas control normal human IgG had no such effect. This finding indicates that EFNB3 costimulation positively regulates an important effector function of T cells, i.e. CTL activity.

Signaling Events in T Cells Stimulated by EFNB3—To understand the mechanisms of EFNB3 costimulation, we first examined the translocation of EFNB3Rs and TCR on the T-cell surface and their relationship to membrane lipid rafts immediately after TCR-crosslinking (Fig. 6). TCR was crosslinked by biotinylated anti-CD3 followed by goat anti-hamster IgG. The TCR complex was stained by streptavidin-Alexa Fluor 594 in red; EFNB3Rs, by EFNB3-Fc followed by anti-human IgG-Alexa Fluor 488 in green; the lipid rafts in the T-cell membrane, by cholera toxin (CT)-Alexa Fluor 594 in red. In resting T cells, rafts, TCR, and EFNB3Rs were evenly distributed throughout the cell surface (0 min). After 10-min crosslinking with anti-CD3, TCR rapidly polarized and formed a cap in one end of the cell. EFNB3Rs also congregated, and they co-localized with TCR (Fig. 6A). After CD3 crosslinking, the rafts underwent congregation and formed caps as well. EFNB3Rs translocated into the raft caps (Fig. 6B). This indicated that the TCR complex (as detected by anti-CD3), EFNB3Rs (as detected by EFNB2-Fc), and the raft (as detected by CT) all clumped together. This provides a morphological base for EFNB3Rs to enhance TCR signaling, because now both TCR and EFNB3Rs are closely associated and located in aggregated rafts, and their respective signaling pathways can interact closely.

In EFNB3-Fc-costimulated T cells, we examined MAPK activation, which is modulated in other cell types when some kinases are activated (38–41). As shown in Fig. 7A, a combination of solid-phase EFNB3-Fc and suboptimal anti-CD3 stimulation for 2 h led to increased p38 MAPK and p44/42 MAPK phosphorylation, a sign of their activation, whereas anti-CD3 at suboptimal concentration had little effect, according to immunoblotting. The membranes were reprobed with anti-p38 MAPK Ab and anti-p44/42 MAPK Ab, respectively. Total p38 MAPK and p44/42 MAPK protein levels were similar with various treatments. Therefore, these MAPKs were activated after EFNB3R-crosslinking. To test whether such MAPK activation was relevant and necessary in EFNB3 costimulation, we used p38 and p44/42 MAPK-specific inhibitors (SB203580 and PD98059, respectively) to treat T cells costimulated by EFNB3. Both inhibitors, but not their nonfunctional structural analogue (SB202474), inhibited EFNB3R stimulation in terms of proliferation (Fig. 7B), indicating that p38 and p44/42 MAPK activation is an integral and necessary part of the EFNB3R signaling pathway.

**DISCUSSION**

We have reported that EFNB3 and EFNB3Rs were expressed in T cells and monocytes/macrophages. T cells costimulated by solid-phase EFNB3-Fc in the presence of suboptimal anti-CD3 showed enhanced proliferation and CTL activity, production of IFN-γ but not IL-2, and expression of certain T-cell activation markers, such as CD69. Upon TCR activation, EFNB3Rs translocated into the raft caps, to which TCR also congregated. Further downstream, MAPK activity was enhanced by EFNB3-Fc costimulation. These results suggest that EFNB3 and its receptors play important roles in T-cell activation and function.

The inherent difficulty in studying the receptors and functions of EFNs is that each of them binds to multiple Eph family members. In the case of EFNB3, it is known to interact with EphA4 (35), EphB3 (36), and EphB4 (37), and its interaction with additional Eph members is also possible. We attempted to identify the receptors mediating the ligation of EFNB3. Soluble EphA4, EphB3, and EphB4 were added to MLC. EphA4 and EphB4, but not EphB3, were able to partially inhibit the T-cell proliferation. This suggests that the former two might mediate the effect of EFNB3. However, as Ephs and EFNs are capable of two-way signaling, and the EphA4 and EphB4 can potentially interact with EFNs in addition to EFNB3, we cannot exclude the possibility that the observed inhibitory effect is due to the binding of EphA4 and EphB4 to certain EFNs (including EFNB3), which transduce negative signals into T cells. Therefore, the receptors responsible for the binding or functions of EFNB3 cannot be conclusively pinpointed until specific mAbs coated with a suboptimal amount of anti-CD3 (0.8 μg/ml) and an optimal amount of anti-CD28 or EFNB3-Fc (both at 10 μg/ml). E. T cells were cultured in wells coated with anti-CD28 (10 μg/ml) or EFNB3 (10 μg/ml), and PMA (20 ng/ml), ionomycin (1 mg/ml), and CyA (250 nM) were added to the culture solution, as indicated. F. T cells were cultured in wells coated with anti-CD3 (0.8 μg/ml), EFNB3 (10 μg/ml), and various concentrations of anti-CD28, as indicated. G. mitomycin C-treated BALB/c spleen cells (4 × 10^6 cells at 200 μl/well) were used as stimulators, and C57BL/6 spleen cells (4 × 10^6 cells at 200 μl/well) were used as responders in MLC. The cells were cultured in the presence of EphA4 (3 μg/ml), EphB3 (3 μg/ml), EphB4 (3 μg/ml), all of the three Ephs (mixture of 3 μg/ml each), NIH3T3 (3 μg/ml), or no additional reagents (Nil). The cells were cultured for 48 h (A–F), or 120 h (G), and their [H]thymidine uptake in the last 16 h was measured. The experiments were performed more than three times and were reproducible. In conjunction with mAbs, “anti” is abbreviated as “a.” All the concentrations indicated in the figure legends represent those employed during the coating procedure, unless indicated otherwise. Means ± S.D. of triplicate samples from a representative experiment are shown.
(and conditional gene knockout mice of) each and every Eph (especially EphBs) become available. However, our study is significant, because the effect of EFNB3 detected here reflects combinatory functions of all receptors triggered by EFNB3, and this is perhaps a true picture of what EFNB3 can achieve in vivo.

Although the EFNB3 signal was initially not found in the spleen by Northern blotting (42), we demonstrated that spleen white pulp expressed EFNB3 mRNA according to in situ hybridization, and this result was supported by detection of EFNB3 expression on T cells and monocytes/macrophages at the protein level by flow cytometry. The discrepancy between the previous report and our current findings is probably because of the sensitivity of the tests employed.

Because EFNB3 is expressed on T cells and monocytes/macrophages implies that all these cells can enhance responsiveness or reduce the response threshold of EFNB3R+ T cells to antigens. This fact might explain the advantage for T cells to be activated in lymphoid organs where leukocytes are tightly packed. Although costimulation from antigen-presenting cells...
to T cells is well studied, we now can understand that the signaling between T-cells is equally important for T-cell activation. A typical proof of this concept can be found in any T-cell culture system, in which minimal cell density is normally required for optimal T-cell activation. Although autocrine is often quoted as a reason for such a requirement, interaction through cell-surface receptors and ligands on the T cells is likely a more fundamental mechanism. Recently, Wang et al. (43) have demonstrated that LIGHT, which is a cell-surface ligand belonging to the tumor necrosis factor family and is primarily expressed on T cells, can stimulate T-cell activation both in vivo and in vitro through its receptor HveA on T cells. We have shown that in pure T-cell culture, the interaction between EphB6 and its T-cell surface ligand can enhance T-cell response to TCR stimulation (20). The expression of both EFNB3 and EFNB3R on T cells and the effect of EFNB3 on T cells costimulation, as documented in this study, suggests the possibility that these two molecules are also involved in T-cell/T-cell co-operation.

We compared T-cell costimulations mediated by CD28 and EFNB3R. The former induced vigorous production of IL-2, IL-4, and IFN-γ, but the latter only triggered IFN-γ secretion, showing a qualitative difference. T cells can be activated experimentally through Ca²⁺-dependent or -independent pathways; the former but not the latter is sensitive to CyA inhibition (33, 34). CD28 costimulation in the presence of PKC activator, PMA, utilizes the Ca²⁺-independent pathway and is resistant to CyA (33, 34). In the same model, EFNB3R costimulation was also resistant to CyA, suggesting that it also activates the Ca²⁺-independent pathway. When anti-CD28 was used at suboptimal but not optimal concentrations, EFNB3 was able to moderately further enhance the T-cell proliferation, suggesting a certain overlap between CD28 and EFNB3R functions. Taken together, these data show that the signaling pathways of CD28 and EFNB3R are distinct but not totally separated.

A recent study showed that plasma membrane compartmentalization plays an essential role in T-cell activation and co-stimulation. On the T-cell membrane, detergent-insoluble glycolipid-enriched rafts contain proteins, such as Src kinases that are highly relevant for T-cell signaling (44). Strong TCR ligation induces the association of TCR and the rafts, and this association is correlated with TCR signaling and T-cell activation (40). In our case, EFNB3R migrated into the rafts after TCR crosslinking (as seen in Fig. 6) by anti-CD3. Therefore, it is possible that EFNB3Rs works in concert with TCR: TCR crosslinking leads to raft reorganization, EFNB3R congregates into the capped rafts where it co-localizes with TCR, and the EFNB3R signaling pathway interacts with that of TCR and enhances the latter.

For proper T-cell activation, Ras and Rac signaling pathways need to be mobilized. Ras activation leads to activation of p44/42 MAPK kinases, which, in turn, results in the synthesis and activation of various transcription factors. On the other hand, activation of Rac and Cdc42 small G proteins elicits p38 MAPK activation, which is essential for cytoskeleton reorganization. Such reorganization is now known to be pivotal for T-cell signaling (40). We have found that the activities of both p44/42 and p38 MAPK are enhanced in the presence of EFNB3 costimulation, and this is consistent with the roles of these MAPK in T-cell activation. Recently, it has been reported that EphB2 activation results in the inhibition of p44/42 MAPK in neuronal cells (45), and that EphA activation leads to inhibition of this kinase in several cell lines of endothelial and epithelial origin (41). Obviously, these reports deal with cell types that are different from those in our study, but the consequence is different as well.

Our study demonstrates the role of EFNB3 in regulating T-cell function. Similar functions were found with EFNB1 and EFNB2 (data not shown), suggesting that these molecules have overlapping but essential functions in T-cell activation. These findings represent an initial step toward understanding the biological significance of ephrins and Ephs in the immune system.
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