Ephrin-B1 Is Critical in T-cell Development*

Guang Yu1, Jianning Mao1, Yulian Wu5, Hongyu Luo1,2, and Jiaping Wu3,4

From the 1Laboratory of Immunology and the 2Nephrology Service of Notre Dame Hospital, Centre Hospitalier de l’Université de Montréal (CHUM), Montréal, Quebec H2L 4M1, Canada and the 3Second Affiliated Hospital of Zhejiang University Medical College, Hangzhou 310009, China

Eph kinases are the largest family of receptor tyrosine kinases, and their ligands, ephrins (EFNs), are also cell surface molecules. In this study, we investigated the role of EFNB1 and the Ephs it interacts with (collectively called EFNB1 receptors) in mouse T-cell development. In the thymus, CD8 single positive (SP) and CD4CD8 double positive (DP) cells expressed high levels of EFNB1 and EFNB1 receptors, whereas CD4 SP cells had moderate expression of both. Soluble EFNB1-Fc in fetal thymus organ culture caused significant subpopulation ratio skew, with increased CD4 SP and CD8 SP and decreased DP percentage, while the cellularity of the thymus remained constant. Moreover, in EFNB1-treated fetal thymus organ culture, CD117+, CD25+, DP, CD4 SP, and CD8 SP cells all had significantly enhanced proliferation history, according to bromodeoxyuridine uptake. In vitro culture of isolated thymocytes revealed that EFNB1-Fc on solid-phase protected thymocytes from anti-CD3-induced apoptosis, with concomitant augmentation of several antiapoptotic factors, particularly in CD4 SP and CD8 SP cells; on the other hand, soluble EFNB1-Fc promoted anti-CD3-induced apoptosis, as was the case in vivo. This study reveals that EFNB1 and EFNB1 receptors are critical in thymocyte development.

Receptors are important cell surface molecules for communication between cells and their environment. Protein-tyrosine kinases are essential components in lymphocyte signaling pathways. Thus, receptor tyrosine kinases have dual pivotal functions in lymphocytes. The Eph kinases are the largest receptor protein-tyrosine kinase family. According to sequence homology, Eph kinases are classified into EphA members (EphA1–EphA9) and EphB members (EphB1–EphB6) (1) (available on the World Wide Web at cbweb.med.harvard.edu/eph-nomenclature/cell_letter.html). Ephrins (EFNs), ligands of Eph kinases, are cell surface molecules as well and can be classified into A and B subfamilies. EFNA members (EFNA1–EFNA6) are glycosylphosphatidylinositol-anchored proteins and bind to EphA members with loose specificity; EFNs (EFNB1–EFNB3) are transmembrane proteins and bind to EphB, again with loose specificity (1). EphA4, an exception, can bind to EFNB2 in addition to EFNA members (2). EFNBs can also function as reciprocal receptors for EphB molecules and reversely transduce signals into cells (3). Most Eph kinases or EFNs have probably already been identified, because sequences from the human genome project have revealed 14 Eph entries and eight EFN entries (4).

Since Eph kinases and their ligands are all cell surface molecules, they can only interact with each other when expressed on adjacent cells. Not surprisingly, the clearly demonstrated function of these receptors and ligands is to control accurate spatial patterning and cell positioning in the central nervous (5, 6) and gastrointestinal (7) systems, in angiogenesis (8), and in urorectal development (9).

Some of the Eph kinases and their ligands are expressed on immune cells (10–13); limited knowledge about their function in immune responses is available and is described as follows. We have reported previously that a kinase-defective Eph family member, EphB6, is capable of transducing signals into T cells, probably through adaptor molecules associated with its intracellular tail (14). Activation of EphB6 with solid-phase anti-EphB6 monoclonal antibody results in Jurkat cell apoptosis (14) or augmentation of normal human T-cell responses to antigen stimulation (15). EphB6+/+ mice have compromised delayed type hypersensitivity and experimental autoimmune encephalitis (16). EFNB1, EFNB2, and EFNB3, as well as their receptors, are expressed on peripheral T cells (17–19). Solid-phase EFNB1, EFNB2, and EFNB3 can augment TCR stimulation in vitro (17–19). Some EphA members have been reported to be expressed on different populations of thymocytes (20), and some EphA and EFNs interfere with T-cell development in thymic organ culture (21). ENFAs can inhibit T-cell chemotaxis (22).

The thymus is the cradle of T cells. Early T-lineage cells in the thymus are usually described as triple negative (TN; CD3+CD4+CD8−) cells. Through the rearrangement of TCR, TN cells become immature double positive (DP, CD4+CD8+) and express a low level of CD3. After this DP transition, the thymocytes undergo extensive positive and negative selections to ensure that the mature T cells are functional and self-tolerant. Positive and negative selection of thymocytes has been reported to occur either in the cortex at the cortex-medullary junction or within the medulla (23–34). After selection, the DP cells transiently express high levels of CD3-associated TCR to form mature DP cells and then rapidly differentiate into mature CD4 or CD8 single positive (SP) T cells that express high levels of TCR (25).

Positive and negative selection depends on major histocompatibility complex (26, 27), on the concentration of peptide presented, and on the accessory molecules involved, all of which are important in determining the strength of TCR signaling (28–30). However, the late stage of positive selection and associated post-selection events seems independent of the peptide-major histocompatibility complex complexes, which are responsible for the initiation of positive selection (31, 32); it is possible that cells adjacent to the T cells under selection express certain cell
FIGURE 1. **EFNB1 and EFNB1R expression in the thymus.** A, *in situ* hybridization analysis of thymus EFNB1 expression. The adult BALB/c thymus was hybridized with DIG-labeled antisense or sense riboprobes of EFNB1. Original magnification was ×10.5. Subcapsule region; C, cortex; M, medulla. B, flow cytometry analysis of EFNB1 and EFNB3 expression or EFNB1R and EFNB3R expression on e17 fetal thymocyte subpopulations. Normal goat IgG and human IgG served as controls for goat anti-EFNB Abs and EFNB-Fc, respectively. These controls are shown as shaded areas; such background staining has been deducted from the percentage shown. The experiment was performed more than three times, and representative histograms are depicted. C, flow cytometry analysis of EFNB1 and EFNB1R on the same subpopulation of thymocytes. Total thymocytes or magnetic bead-purified CD4 SP and CD8 SP thymocytes from 5-day-old neonatal mice were stained with anti-EFNB1 and EFNB1-Fc; normal goat IgG and human IgG were used as controls for anti-EFNB1 and EFNB1-Fc, respectively. The experiment was performed twice, and representative histograms are shown.
EphrinB1 Regulates Thymocyte Survival

surface-associated molecules that provide additional signals needed for this stage.

In this study, we investigated the expression of EFNB1 and its receptors in thymocytes and its role in modulating T-cell development.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—A 534-bp cDNA fragment of mouse EFNB1 cDNA from position 336 to 890 (accession number U12983) was fetched with PCR from a mouse embryonic tissue cDNA library and cloned into pGEM-4Z (Invitrogen). The resulting construct, pGEM-4Z-mB1, served to transcribe antisense probes with SP6 RNA polymerase or to transcribe sense probes with T7 RNA polymerase, using digoxigenin RNA labeling kits (Roche Applied Science). In situ hybridization was carried out according to instructions from the kit manufacturer.

Fetal Thymus Organ Culture (FTOC)—Thymuses were sterilely isolated from embryonic day 17 (e17) BALB/c fetuses and cultured in FTOC medium at 37 °C in 5% CO₂, as described by DeLuca et al. (33). Briefly, the isolated thymic lobes were placed on the surface of a white plain Millipore (Bedford, MA) filter membrane (ISOPORE; 0.4-μm pore size), which was supported underneath by surgical gel foam (ETHICON, Somerville, NJ) in 24-well plates (Costar, Corning, NY). Each well contained 0.8 ml of culture medium consisting of Dulbecco’s modified Eagle’s medium, 20% fetal calf serum (Hyclone Laboratories, Logan, UT), streptomycin (100 μg/ml), and penicillin (250 units/ml). Nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (20 mM), and 3.4 mg/ml sodium bicarbonate were added to the medium as supplements. EFNB1-Fc (19), EFNB2-Fc (17), EFNB3-Fc (18), normal human IgG, and BrdUrd were also included in the medium as indicated in the different experiments. Half of the medium was replaced every 2–3 days until harvesting.

Flow Cytometry—For the measurement of EFNB and EFNB receptor expression in different thymocyte subpopulations and of the percentage of different thymocyte subpopulations, thymocytes from fresh e17 thymuses or from FTOC were stained with EFNB-Fc/goat anti-human IgG-FITC or with goat anti-EFNB (R&D Systems, Minneapolis, MN), followed by donkey anti-goat IgG-FITC (Cedarlane, Hornby, Ontario, Canada); anti-CD4-biotin/streptavidin-Quantum Red and anti-CD8-PE, anti-CD25-PE, or anti-CD117-PE (PharMingen, San Diego, CA) were employed to gate different thymic subpopulations. To assess cell proliferation, anti-BrdUrd-Alexa 488 (Molecular Probes, Inc., Eugene, OR) was used for staining. Annexin V-FITC (PharMingen) was used to stain apoptotic cells.

Reverse Transcription-Real Time PCR—Total RNA from cultured thymocytes was extracted with Trizol (Invitrogen) and reverse transcribed with Omniscript reverse transcription kits from Qiagen (Mississauga, Ontario, Canada). Real time PCR was performed using Quanti T Test SYBR Green PCR kits (Qiagen). The primers for Bcl-2 were 5′-ctgtcactcgctgcggactc and 5′-gtgatggactgctggctactcagc; for primers for Bcl-XL they were 5′-iggtgatgaactgggagcactcagtgtgcaag and 5′-agcasagctgctgcggcactcaag; for primers for Flip they were 5′-gtgatggactgctggctactcagc and 5′-agcasagctgctgcggcactcaag; and for primers for β-actin they were 5′-tctgcacagacgagttgaga and 5′-tattgcagcagactcttt. The PCR program started with 15 min of holding at 95 °C, followed by 40 cycles of amplification: denaturing, 15 s at 94 °C; annealing, 30 s at 55 °C; extension, 30 s at 72 °C. PCR was conducted in triplicate, and signal ratios (mean ± S.D.) of Bcl-2 versus actin, Bcl-XL versus actin, and Flip versus actin from their linear amplification stage were presented.

RESULTS

EFNB1 and EFNB1R Expression in the Thymus—Expression of EFNB1 mRNA in the adult thymus was detected by in situ hybridization. As shown in Fig. 1A, the cortex was highly positive; sparse signals were present in the medulla; and the subcapsule region was negative. Expression of EFNB1 and its receptors on various thymocyte subpopulations at the protein level was then investigated by flow cytometry. The adult and e17 thymus had similar expression patterns, and representative histograms of e17 thymocytes are shown in Fig. 1B. CD8 SP and CD4CD8 DP cells had the highest percentage of EFNB1-positive cells at 29.9 and 29.4%, respectively (upper row of Fig. 1B); DN (double negative) cells had a lower percentage (13.5%); CD4 SP cells had the lowest (6.2%). In comparison, another EFNB family member, EFNB3 (upper row of Fig. 1B), had a low percentage of positive cells in all of these subpopulations (3.3% in DN, 1.2% in DP, 2.3% in CD4 SP, and 7.4% in CD8 SP). Such differential expression of EFNB1 versus EFNB3 suggests that the former plays a more important role in T-cell development in the thymus.

EFNB1 promiscuously binds to multiple Eph family members (2, 34). The receptor binding of EFNB1 to thymocytes was assessed by EFNB1-Fc staining, which was produced as a recombinant molecule between the EFNB1 extracellular sequence and the human IgG Fc sequence with the Fc receptor binding site mutated, as described in our previous publication (19); such binding revealed the interaction of EFNB1 with the ensemble of all of its receptors, which are collectively referred to as EFNB1R, on thymocytes. As shown in the lower row of Fig. 1B, the percentage of EFNB1R-positive cells was highest in CD8 SP (40.7%), followed by DP (31.5%) and CD4 SP (8.5%); DN contained no EFNB1R-positive cells (0%). In contrast, the percentage of EFNB3R-positive cells (lower row of Fig. 1B), as measured by EFNB3-Fc (18) binding, was low in most subpopulations (0% in DN, 3.2% in DP, and 0.3% in CD4 SP), with the exception of CD8 SP (11.4%), suggesting a more prominent role of EFNB1R than that of EFNB3 receptors in thymic T-cell development.

We next asked whether EFNB1 and EFNB1R were expressed in the same or different cells. As shown in Fig. 1C, in CD4 SP, CD8 SP, or total thymocytes, among which 80% were DP cells, EFNB1 and EFNB1R expression was a continuum, with some cells expressing EFNB1 alone, some cells expressing EFNB1R alone, and some cells expressing both.

It is worth mentioning that we have previously reported that about 30% of CD4 and CD8 cells in the periphery express EFNB1 and EFNB1R (19). Soluble EFNB1 Disturbs Subpopulation Ratios in FTOC—FTOC was employed to investigate the role of EFNB1 and EFNB1R interaction in thymocyte development in a setting relatively close to a physiological condition; various recombinant proteins were added to the culture. We first verified whether EFNB1-Fc could enter the thymus easily. FTOC was conducted in the presence of EFNB1-Fc (10 μg/ml) for 24 h; thymocytes were flushed out, extensively washed, and then reacted with PE-conjugated anti-human IgG; 31.3% of the thymocytes were positive for EFNB1R (Fig. 2A, right), and this percentage was comparable with that of fresh thymocytes stained with EFNB1-Fc followed by PE-conjugated anti-human IgG (Fig. 2A, left). This indicates that EFNB1-Fc can effectively enter the FTOC within 24 h.

Next, the effect of EFNBs on FTOC was investigated. We examined FTOC on different time points, such as days 5, 7, and 10, and changes in thymocyte subpopulations in EFNB1-Fc-treated FTOC were not apparent before day 10 (data not shown). Thus, all of the rest of the experiments were conducted with 10-day FTOC. EFNB1-Fc, EFNB2-Fc, EFNB3-Fc, or NHIgG (all at 10 μg/ml) was present in culture, and the CD4 and CD8 expression of cells from FTOC was assessed by flow cytometry. As shown in Fig. 2B, in comparison with thymocytes from NHIgG-treated FTOC, there was a consistent and obvious decrease of DP (from 69.9 to 37.2%) but an increase of CD4 SP (from 16.0 to 30.1%) and CD8 SP (from 5.4 to 10.9%) percentage. On the other hand, EFNB2-Fc and EFNB3-Fc did not drasti-
cally alter the ratios of these subpopulations. Therefore, for the rest of this study, our attention was focused on EFNB1 and EFNB1R; EFNB3-Fc was used for comparison only.

The ratios of thymocyte subpopulations were determined by a combination of factors, such as proliferation, apoptosis, and extinguishing of CD4 or CD8 expression on DP cells. To gain insight into the reasons for the observed ratio disturbance in EFNB1-Fc-treated FTOC, we measured the proliferation history of each subpopulation by BrdUrd uptake during the 10-day culture period (Fig. 3A). There was a significant increase of BrdUrd-positive cell percentage in DN (from 31.7 to 61.3%), DP (from 11.3 to 54.5%), CD4 SP (from 10.2 to 48.0%), and CD8 SP (from 4.4 to 89.7%) subpopulations in EFNB1-Fc compared with that of NHIgG-treated FTOC. In DN cells, CD117⁺ cells represent the earliest subpopulation, followed by CD25⁺ cells (35); both...
of these subpopulations had significantly increased proliferation history in the presence of EFNB1-Fc, in comparison with NHIgG (from 17.4 to 53.6% for CD117⁺/H11001 and from 31.7 to 63.2% for CD25⁺/H11001). EFNB3-Fc treatment increased the BrdUrd-positive percentage in these subpopulations to a much lesser extent, consistent with the lower degree of EFNB3 and EFNB3R expression in thymocytes.

It is to be noted that the thymocytes continuously differentiate from CD117⁺/H11001 CD25⁺/H11002 DN to CD117⁺/H11002 CD25⁺/H11001 DN to DP to CD4⁺/H11001 SP or CD8⁺/H11001 SP cells; therefore, BrdUrd in cells of a later stage could be inherited from their predecessors or incorporated from proliferation during the said stage or both. Regardless, this experiment clearly demonstrated that soluble EFNB1-Fc enhanced thymocyte proliferation.

What is the effect of the increased proliferation on thymocyte cellularity? The results (Fig. 3B) revealed that the numbers were similar in EFNB1-Fc-, EFNB3-Fc-, or NHIgG-treated FTOC after 5, 10, or 12
days. This suggests that there must be increased apoptosis in situ in FTOC to keep the constant cellularity in the presence of enhanced thymocyte proliferation after soluble EFNB1-Fc treatment.

**EFNB1 and EFNB1R Interaction Regulates Thymocyte Survival**—It is difficult to assess the actual rates of apoptosis of thymocyte subpopulations in FTOC, because apoptotic cells are constantly and rapidly removed, and the efficiency of this process cannot be accurately determined. We therefore employed an in vitro system, in which the apoptotic cell removal was no longer effective, to assess the effect of EFNB1 on thymocyte apoptosis. In FTOC, soluble EFNB1 probably blocked the natural interaction between EFNB1 and its receptors, both of which were present on thymocyte subpopulations examined. This implied that thymocytes would normally receive survival signals through their EFNB1 receptors by interacting with solid-phase EFNB1 expressed on neighboring thymocytes or stroma cells. To prove this point, we cultured e20 thymocytes in wells coated with EFNB1 or control NHlgG (10 μg/ml for coating in both cases), and thymocyte apoptosis was induced by solid-phase anti-CD3 (5 μg/ml for coating). As shown in Fig. 4A, 4B, and 4C, DP, CD4 SP, and CD8 SP cells in control samples suffered apoptosis at 45.0, 29.4, and 28.8%, respectively, after overnight culture; in contrast, the apoptosis of these cells decreased to 33.7, 15.2, and 21.4%, respectively, in the presence of solid-phase EFNB1-Fc. This result was very reproducible in more than eight experiments and clearly demonstrated that when EFNB1R on thymocytes were cross-linked, the cells received survival signals.

Since cross-linking EFNB1R could promote thymocyte survival, it followed that soluble EFNB1-Fc should interfere with the endogenous interaction between EFNB1 and EFNB1R on fraternal thymocytes, disrupt the survival signals delivered to EFNB1R, and increase apoptosis. This possibility was tested in vitro using thymocytes from 1-day-old mice (Fig. 3B). The cells were cultured in the presence of suboptimal soluble anti-CD3 to trigger minimal apoptosis (30.2% in DP cells, 6.9% in CD4 SP cells, and 10.9% in CD8 SP cells). In the presence of soluble EFNB1-Fc, the percentage of apoptotic cells was consistently increased in all of the three subpopulations (40.5% in DP cells, 16.1% in CD4 SP cells, and 14.5% in CD8 SP cells). Again, such an effect was very reproducible and conversely corroborated the findings above based on solid-phase EFNB1-Fc.

To elucidate the survival mechanism, we tested the expression of several antiapoptotic factors, such as Bcl-2, Bcl-XL, and Flp (36, 37), in these cells. DP, CD4 SP, and CD8 SP cells from e20 thymuses were sorted by flow cytometry and then cultured in the presence of solid-phase anti-CD3 and EFNB1-Fc, as described above. After overnight culture, the cells were harvested, and their Bcl-2, Bcl-XL, and Flp mRNA levels were assayed with reverse transcription–real time PCR (Fig. 4C). Cells treated with solid-phase anti-CD3 and NHlgG were used as controls. In the EFNB1–treated CD4 SP cells, mRNA of Bcl-2, Bcl-XL, and Flp was augmented; in CD8 SP cells, mRNA of the former two molecules was increased, whereas Flp mRNA remained unchanged. This result is, in general, consistent with the antiapoptotic effects of these molecules and the observed reduction of apoptosis in these cells. We noticed that CD4 SP and CD8 SP had different levels of Bcl-2 and Bcl-XL; also, Flp mRNA in CD8 cells was not modulated by solid-phase EFNB1. This suggests differential involvement of these molecules in the protection of CD4 SP and CD8 SP cells. The results from DP cells were perplexing. Despite the fact that these cells were protected by solid-phase EFNB1-Fc from apoptosis, their Bcl-2 and Flp mRNA and, to a lesser extent, Bcl-XL mRNA were actually decreased; this has raised an intriguing possibility that the balance of pro- and antiapoptotic factors in these cells is differentially regulated compared with CD4 SP and CD8 SP cells, and antiapoptotic factors other than those examined here are responsible for the protection of DP cells.

**DISCUSSION**

In this study, we showed that EFNB1 and EFNB1R were expressed differentially on thymocyte subpopulations; FTOC in the presence of soluble EFNB1-Fc had significant subpopulation ratio skew, possibly the result of disturbance due to apoptosis and proliferation of different subpopulations; solid-phase EFNB1 delivered survival signals via EFNB1R to the thymocytes, accompanied by modulation of certain antiapoptotic factors; and soluble EFNB1 promoted thymocyte apoptosis. These results suggest that EFNB1 and its receptors play important roles in thymocyte TCR signaling strength, which modulates thymocyte survival.

When soluble EFNB1-Fc was present in FTOC, it caused a drastic reduction of DP cells and an increase of CD4 and CD8 SP cells. The changes were in the percentage of each subpopulation, since the total cellularity remained similar in the EFNB1-Fc- versus NHlgG-treated thymus. We observed that there was enhanced proliferation history in all of the subpopulations tested, and at the same time, we demonstrated that solid-phase EFNB1 promoted thymocyte survival, and soluble EFNB1 enhanced their apoptosis. Based on these observations, two alternative models could be proposed.

In the first model, soluble EFNB1 enters the thymus and drives the proliferation of thymocyte subpopulations; such proliferation, in turn, triggers a homeostatic mechanism to maintain constant thymic cellularity. As a result, excess thymocytes were eliminated by apoptosis; the imbalance between proliferation and apoptosis was manifested as altered ratios of subpopulations in FTOC. However, we found no evidence that soluble EFNB1-Fc could stimulate isolated thymocytes to proliferate in vitro, in the absence or presence of TCR stimulation (data not shown). Moreover, although DN cells expressed no EFNB1R, they still had significantly increased proliferation in EFNB1-Fc- but not NHlgG-treated FTOC. The validity of this model is thus very questionable.

In the second model, the soluble EFNB1-Fc causes apoptosis of thymocytes; the consequent loss of thymocytes in FTOC triggers compensatory proliferation to maintain homeostatic cellularity in the thymus. The imbalance of apoptosis and proliferation results in subpopulation ratio skew. This model is more appealing, because we showed that in vitro, solid-phase EFNB1 inhibited DP, CD4 SP, and CD8 SP thymocyte apoptosis, whereas soluble EFNB1-Fc promoted apoptosis of these cells. This suggests that in vivo, the soluble EFNB1-Fc might block the physiological role of cell surface EFNB1 in promoting survival of neighboring EFNB1R-bearing thymocytes by delivering antiapoptotic signals. This conclusion is supported by a previous study by Roifman’s group (38). We demonstrated that total thymocytes, CD4 SP, and CD8 SP cells could all be EFNB1-positive alone or EFNB1R-positive alone or bear both EFNB1 and EFNB1R at the same time on an individual cell (Fig. 1C); we also demonstrated that the survival of all of these cell subpopulations was affected by solid-phase and soluble EFNB1. Taken together, such observations suggest that EFNB1 on a given subpopulation of thymocytes is capable of acting in cis within the said subpopulation as well as in trans on another subpopulation to modulate thymocyte survival.

In the thymus, most apoptosis occurred in DP cells, which have a 25–33% daily death rate, with 97% of them eventually undergoing apoptosis due to the lack of positive selection (39). The precursors of DP cells (i.e. DN of various stages) vigorously proliferate, but their apoptosis is not obvious. Although it is commonly believed that negative selection occurs at the DP stage (40), Baldwin et al. (24) reported that it could happen throughout thymic development at DN, DP, and SP stages. This implies that some DN and SP cells also undergo apoptosis. The majority
of SP cells are located in medulla. The number of cells entering into and exiting from the medullar daily is about the same. The medullary cells proliferate at a low rate of 2–5% (41). Taken together, this indicates that daily apoptosis in SP cells, even with the presence of negative selection, should be no more than 2–5% to maintain a balanced budget. Our data suggest that endogenous EFNB1R-EFNB1 interaction lowers the TCR response threshold (see below). Obviously, soluble EFNB1-Fc can interrupt such interaction and raise the threshold, resulting in more cells without receiving a sufficient positive selection signal at the DP stage; this is in keeping with fact that the DP population was significantly reduced in FTOC after EFNB1 treatment.

How do we explain that DP had increased BrdUrd uptake, yet their percentages in EFNB1-treated FTOC were significantly reduced? First of all, as mentioned under “Results,” BrdUrd uptake of DP in FTOC reflected the proliferation history of DP and all of its predecessors in the precedent DN stage and did not necessarily represent the proliferation of all, as mentioned under “Results,” BrdUrd uptake of DP in FTOC. This is in keeping with fact that the DP population was significantly reduced in FTOC after EFNB1 treatment.

Moreover, no rapid conversion of DP to SP cells was found in isolated EFNB1-expressing cells to enhance the survival of SP cells, but they probably need to work in concert with TCR for such an effect. Our data clearly showed that EFNB1 and its receptors are important in thymocyte survival and development.

Acknowledgment—We thank Ovid Da Silva (Research Support Office, Research Center, CHULM, Montreal, Canada) for editorial assistance.

REFERENCES
1. Eph Nomenclature Committee (1997) Cell 90, 403–404
2. Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenmiken, A., Pan, L., Ryn, T. E., Henkemeyer, M., Strebbhardt, K., Hirai, H., Wilkinson, D. G., Pawson, T., Davis, S., and Yancopoulos, G. D. (1996) Nature 383, 722–725
3. Gale, N. W., Mhamalou, G., Yancopoulos, G. D., Henkemeyer, M., and Pawson, T. (1996) Nature 383, 722–725
4. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Houssaint, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zhang, X. H., Chen, L., Grass, C. M., Grimrudian, J. Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Flora, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Moharry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazza, V., Brandon, R., Cargill, M., Chan-dramouliswaran, I., Charlah, R., Chaturvedi, K., Deng, Z., Di F., Dunn, P., Eilbek, K., Evangelista, C., Gabrielian, A. E., Gao, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. B., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, L., Liu, F., Merkulov, G. V., Mishina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, S., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Widres, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhang, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhou, S., Gilbert, D., Buerker, S., Spier, G., Carter, S., Cravchik, A., Woodage, T., Ali, F., An, H., Awei, A., Baldwin, D., Baden, H., Barning, M., Barlow, I., Beeson, K., Baxman, D., Carver, A., Cheng, M., Curry, L., Danaher, S., Davenport, L., Deslits, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Gagn, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostein, D., Housk, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Kodurui, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMillan, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Freis, P., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhiel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Tseh, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zarevi, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M. J., Sjolander, K. V., Karab, K., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Baftin, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Bick, L., Chimnha, M., Caron-Stine, I., Caukh, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A., Domroscki, M., Donnelly, M., Dyer, E., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glorek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., and Nodel, M. (2001) Science 291, 1304–1354
5. Wilkinson, D. G. (2000) Int. Rev. Cytol. 196, 177–244
6. Flanagan, J. G., and Vanderhaeghen, P. (1998) Annu. Rev. Neurosci. 21, 309–345
7. Pastore, D. P., and Goodboy, D. (1999) J. Clin. Invest. 114, 1151–1160
8. Pastore, D. P., and Goodboy, D. (1999) J. Clin. Invest. 114, 1151–1160
18. Yu, G., Luo, H., Wu, Y., and Wu, J. (2003) *J. Biol. Chem.* 278, 47209–47216
19. Yu, G., Luo, H., Wu, Y., and Wu, J. (2004) *J. Biol. Chem.* 279, 55531–55539
20. Vergara-Silva, A., Schaefer, K. L., and Berg, L. I. (2002) *Gene Expr. Patterns* 2, 261–265
21. Munoz, J. J., Alonso, C., Sacedon, R., Crompton, T., Vicente, A., Jimenez, E., Varas, A., and Zapata, A. G. (2002) *J. Immunol.* 169, 177–184
22. Sharfe, N., Freywald, A., Toro, A., Dadi, H., and Roifman, C. (2002) *Eur. J. Immunol.* 32, 3745–3755
23. Murphy, K. M., Heimberger, A. B., and Loh, D. Y. (1990) *Science* 250, 1720–1723
24. Baldwin, K. K., Trenchak, B. P., Altman, J. D., and Davis, M. M. (1999) *J. Immunol.* 163, 689–698
25. Middlebrook, A. J., Martina, C., Chang, Y., Lukas, R. J., and DeLuca, D. (2002) *J. Immunol.* 169, 2915–2924
26. Arnesen, D., and Kruthbeek, A. M. (1998) *Immunol. Rev.* 165, 209–229
27. Anderson, G., and Jenkinson, E. (1997) *Immunol. Today* 18, 363–364
28. Ashton-Rickardt, P. G., and Tonegawa, S. (1994) *Immunol. Today* 15, 362–366
29. Ober, B. T., Hu, Q., Opferman, J. T., Hagevik, S., Chiu, N., Wang, C. R., and Ashton-Rickardt, P. G. (2000) *Int. Immunol.* 12, 1353–1363
30. Stefanski, H. E., Mayerova, D., Jameson, S. C., and Hogquist, K. A. (2001) *J. Immunol.* 166, 6602–6607
31. Hare, K. J., Jenkinson, E. J., and Anderson, G. (1999) *J. Immunol.* 162, 3978–3983
32. Hare, K. J., Jenkinson, E. J., and Anderson, G. (2001) *Cell Mol. Biol. (Noisy-Le-Grand)* 47, 119–127
33. DeLuca, D., Bluestone, J. A., Shultz, L. D., Sharrow, S. O., and Tatsumi, Y. (1995) *J. Immunol. Methods* 178, 13–29
34. Prevoet, N., Woulfe, D. S., Tognolini, M., Tanaka, T., Jian, W., Fortina, R. R., Jiang, H., and Brass, L. F. (2004) *Blood* 103, 1348–1355
35. Varas, A., Hager-Theodorides, A. L., Sacedon, R., Vicente, A., Zapata, A. G., and Crompton, T. (2003) *Trends Immunol.* 24, 197–206
36. Kim, R. (2005) *Biochem. Biophys. Res. Commun.* 333, 336–343
37. Kataoka, T. (2005) *Crit. Rev. Immunol.* 25, 31–58
38. Freywald, A., Sharfe, N., Rashotte, C., Grunberger, T., and Roifman, C. M. (2003) *J. Biol. Chem.* 278, 10150–10156
39. Werlen, G., Hausmann, B., Naeher, D., and Palmer, E. (2003) *Science* 299, 1859–1863
40. Srb, C. D., and Sprent, J. (1994) *Nature* 372, 100–103
41. Scollay, R., and Godfrey, D. I. (1995) *Immunol. Today* 16, 268–273
42. Viret, C., and Janeway, C. A., Jr. (1999) *Rev. Immunogenet.* 1, 91–104
43. Germain, R. N. (2003) *Immunol. Rev.* 277, 277–286
44. von Boehmer, H., Aifantis, I., Gounari, F., Azogui, O., Haughn, L., Apostolou, I., Jaeckel, E., Grassi F., and Klein, L. (2003) *Immunol. Rev.* 191, 62–78