Efnb1 and Efnb2 Proteins Regulate Thymocyte Development, Peripheral T Cell Differentiation, and Antiviral Immune Responses and Are Essential for Interleukin-6 (IL-6) Signaling\textsuperscript{*\textcircled{S}}

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**Background:** Ephrins (Efns) are the ligands of Eph kinases. The roles of Efns in the T cell compartment are studied.

**Results:** Efnb1 and Efnb2 double knock-out mice showed compromised thymocyte development, Th1 and Th17 function, IL-6 receptor signaling, and antiviral responses.

**Conclusion:** Efnb1 and Efnb2 are involved in the T cell development and function.

**Significance:** This study has revealed novel biological roles of Efns.

Ephrins (Efns), are also cell surface molecules. Because of promiscuous interaction between Ephs and efns, there is considerable redundancy in this system, reflecting the essential roles of these molecules in the biological system through evolution. In this study, both Efnb1 and Efnb2 were null-mutated in the T cell compartment of mice through loxP-mediated gene deletion. Mice with this double conditional mutation (double KO mice) showed reduced thymus and spleen size and cellularity. There was a significant decrease in the DN4, double positive, and single positive thymocyte subpopulations and mature CD4 and CD8 cells in the periphery. dKO thymocytes and peripheral T cells failed to compete with their WT counterparts in irradiated recipients, and the T cells showed compromised ability of homeostatic expansion. dKO naive T cells were inferior in differentiating into Th1 and Th17 effectors \textit{in vitro}. The dKO mice showed diminished immune response against LCMV infection. Mechanistic studies revealed that IL-6 signaling in dKO T cells was compromised, in terms of abated induction of STAT3 phosphorylation upon IL-6 stimulation. This defect likely contributed to the observed \textit{in vitro} and \textit{in vivo} phenotype in dKO mice. This study revealed novel roles of Efnb1 and Efnb2 in T cell development and function.

Efns are the largest family of cell surface receptor tyrosine kinases, comprising about 25% of known receptor tyrosine kinases (1). There are a total of 15 Efns that are classified into A and B subfamilies according to their sequence homology; the former has nine members and the latter six, although not all are expressed in a given species (2, 3). The ligands of Efns, ephrins (Efns), are also cell surface molecules (1). There are nine Efns divided into A and B subfamilies according to the way they anchor to the cell surface. The Efna subfamily has six members that are glycosylphosphatidylinositol-anchored membrane proteins; the Efnb subfamily has three members that are transmembrane proteins. Interactions between Ephs and Efns are promiscuous. One Eph can interact with multiple Efns and vice versa. In general, Eph members preferentially interact with Efna members and Ephb members with Efnb members (2–4). Such promiscuous interactions suggest that these molecules are so vital to biological systems that heavy redundancy is essential.

Ephs\textsuperscript{3} are receptor tyrosine kinases that can initiate signal transduction upon ligand binding. However, it is known that although Efns are ligands, they can also transduce signals into cells (2, 3) in a phenomenon called reverse signaling. As a result, the interaction between Eph and Efns results in bi-directional signaling. Because Ephs and Efns are cell surface molecules,
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they will normally be activated locally by their binding partners expressed on opposing cells during physical contact. As a result, the major functions of Eph and Efn are related to pattern formation; however, additional functions not related to pattern formation have been recently observed.

Most reported functions of Ephs occur in the central nervous system where they are expressed in neurons and control axon and dendrite positioning (2, 3). They are essential in the development of neuronal connections, circuit plasticity, and repair. However, they are also involved in a variety of other processes. Some Ephs and Efns are expressed in endothelial cells and are vital in angiogenesis during normal embryonic development as well as in tumorigenesis (4, 5). Studies have shown that 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 (6). It has been reported that Ephb2 and Efnb2 are expressed on the endoderm during embryonic development and that their bidirectional interaction is essential in urorectal development (7).

Pancreatic β-cells communicate with each other via Epha and Efn family members to synchronize their insulin secretion in response to blood glucose fluctuations (8). Several Ephb and Efnb family members are expressed on osteoclasts and osteoblasts where they regulate bone development, maintenance, and repair (9, 10). Multiple Eph and Efn members have been found to be expressed in some cancer cells, and they seem to influence cancer cell growth (11). Ephb4 and Efnb2 are expressed on hematopoietic progenitor cells and regulate red blood cell production in response to hypoxia (12). Efnb1 and Eph4 expression in platelets contributes to the clotting process (13). Ephb1 expression on kidney epithelial cells (podocytes) likely plays a role in glomerular filtration (14). The interaction between Ephb2 and Efnb2 regulates the ionic homeostasis of vestibular endolymph fluid in the inner ear (15).

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 and are capable of modulating T cell responses and survival (16–24). We have shown that Efnb1, Efnb2, and Efnb3 initiate signaling through their Eph receptors and can costimulate peripheral T cells in terms of enhancing cytokine production and proliferation *in vitro* (25–27). We further demonstrated that one of these Efnb receptors, Ephb6, although lacking kinase activity, can transmit signals into T cells (28) and that its null mutation leads to compromised T cell responses *in vitro* and *in vivo* (29). However, Ephb6 null mutants have normal thymus structure and thymocyte development (29), probably due to complementary functions of other Eph family members. To reveal the roles of these highly redundant Eph/Efn family members in thymocyte development and T cell immune responses *in vivo* and *in vitro*, we generated Efnb1/Efnb2 double gene knock-out (dKO) mice with T cell-specific deletion of these two genes. Results obtained from these mice showed that Efnb1 and Efnb2 are involved in proper thymocyte development and peripheral T cell function.

**MATERIALS AND METHODS**

*Generation of T Cell-specific Efnb1 and Efnb2 Gene Knockout Mice*—A PCR fragment amplified with a primer set (5′-CTGAATAAGGGCTGCAAAG-3′ and 5′-GCAAATGGCCTAACCCAAGA-3′) based on the Efnb1 genomic sequence was used as a probe to isolate a genomic BAC DNA clone 4M20 from the 129/sv mouse BAC genomic library RPCI-22. A genomic BAC DNA clone 85F06PCR fragment based on the Efnb2 sequence was similarly amplified with the primer set (5′-GCTTGCTCTTCTAGTCAAGGCTGCAAAGA-3′ and 5′-TACCAAGTCTACCAACAGGCA-3′). The targeting vectors were constructed by recombination and routine cloning methods using a 12-kb *Efnb1* genomic fragment from clone 4M20 for *Efnb1* and a 12-kb *Efnb2* genomic fragment from clone 85F06 for *Efnb2* (illustrated in Fig. 1, A and E). The final targeting fragments for *Efnb1* and *Efnb2* were excised from its cloning vector backbone by NotI and electroporated into ES cells. After G418 selection, the FRT-flanked *Neo/TK* cassette was eliminated by subsequent transient transfection of the ES cells with a Flippase expression vector. The targeting scheme is shown in Fig. 1A. These genetic engineering steps in ES cells resulted in two net insertions for the Efnb1 constructs as follows: a 118-bp LoxP-containing sequence (5′-AGTACGGGCC CAAGCTGGCCGCCCTAGGGGGCGCGCTAAGGCTGCAAAGA-3′) and a 151-bp LoxP plus FRT-containing sequence (5′-AGTACGGGCC CAAGCTGGCCGCCCTAGGGGGCGCGCTAAGGCTGCAAAGA-3′), resulting in a 12-kb genomic fragment from clone 4M20 for *Efnb1* and a 12-kb *Efnb2* genomic fragment from clone 85F06 for *Efnb2*.

**RESULTS**

The targeted ES cell clones were injected into C57BL/6 blastocysts. Chimeric male mice were mated with C57BL/6 females to establish mutated *Efnb1* and *Efnb2* allele germ line transmission. Southern blotting with a probe corresponding to the 3′ sequences outside the targeting region, as shown in Fig. 1A (black square), was used to screen and confirm the gene targeting and the successful removal of the Neo-TK selection marker in *Efnb1* ES cells and eventually in mouse tail DNA. The targeted allele showed a 7.3-kb Asel/Scal fragment, whereas the WT allele showed an 11.6-kb fragment (Fig. 1B). Southern blotting with a probe corresponding to the 5′ sequences outside the
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Targeting region, as illustrated in Fig. 1E (black square), was used to screen and confirm the gene targeting and the successful removal of the Neo-TK selection marker in Efnb2 ES cells and eventually in mouse tail DNA. The targeted allele showed an 8.1-kb PacI/PacI fragment in tail DNA (Fig. 1F), although a WT allele showed a 15.5-kb fragment. PCR was used for routine genotyping of the targeted allele(s). The following PCR conditions were used for Efnb1: 4 min at 95 °C, followed by 34 cycles of 15 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C, and a final incubation at 72 °C for 10 min. Primers 5′-GTCTC CACTG CCCAT AGCTC-3′ (forward) and 5′-TGCTC CCAGT CCAGT ACTA-3′ (reverse) amplified a 271-bp fragment from the targeted allele and a 133-bp fragment from the WT allele. PCR conditions for Efnb2 were similar except the annealing temperature was reduced from 58 to 52 °C. Primers 5′-TAGCC ATCCCA TTGGA ATACG-3′ (forward) and 5′-TTGGCC GGCAG CCTTT CGAAG-3′ (reverse) amplified a 456-bp fragment from the targeted allele and a 355-bp fragment from the WT allele.

Mice with targeted Efnb1 allele(s) were named Efnb1<sup>LoxP/LoxP</sup> (loxP insertions in both alleles) or Efnb1<sup>LoxP/Cre</sup> (loxP insertion in one allele). Mice with targeted Efnb2 allele(s) were named Efnb2<sup>LoxP/LoxP</sup> (loxP insertion in both alleles). These two lines were backcrossed with C57BL/6 for more than 10 generations and then mated with Lck promoter-driven Cre recombinase transgenic mice in the C57BL/6 background (strain B6.Cg-Tg (Lck-Cre) 540) to obtain T cell-specific Efnb1 and Efnb2 gene knock-out mice (Lck-Cre-Efnb1<sup>LoxP/LoxP</sup> and Efnb2<sup>LoxP/LoxP</sup> gene knock-out mice (Lck-Cre-Efnb2<sup>LoxP/LoxP</sup>)). Because Efnb1 is an X-linked gene, Lck-Cre-Efnb1<sup>LoxP/LoxP</sup> females are equivalent to Lck-Cre-Efnb1<sup>LoxP/Cre</sup> females in that the Efnb1 gene is completely null-mutated. For the convenience of description, Lck-Cre-Efnb1<sup>LoxP/LoxP</sup> males and Lck-Cre-Efnb1<sup>LoxP/Cre</sup> females are collectively referred to as Lck-Cre-Efnb1<sup>LoxP/Cre</sup> mice. PCR was used to confirm the T cell-specific deletion of Efnb1 using two different primer pairs. Primer pair 1 (forward, 5′-GTCTC CACTG CCCAT AGCTC-3′, and reverse, 5′-TGCTC CCAGT CCAGT ACTA-3′) was used to detect a 271-bp fragment derived from an allele with deleted Exon 1. Primer pair 2 (forward, 5′-GTCTC CACTG CCCAT AGCTC-3′, and reverse, 5′-ACCT TACAT CGAAG AACTG GGCA-3′) was used to detect a 492-bp fragment derived from an allele with exon 1 deleted. Similarly, T cell-specific deletion of Efnb2 was confirmed using two different primer pairs. Primer pair 1 (forward, 5′-TAGCC ATCCCA TTGGA ATACG-3′, and reverse, 5′-TTGGCC GGCAG CCTTT CGAAG-3′) was used to detect a 456-bp fragment derived from an allele(s) with undeleted Exon 1. Primer pair 2 (forward, 5′-CTAAG GTCTC CAGCT CGGTG-3′, and reverse, 5′-TTGGCC GGCAG CCTTT CGAAG-3′) was used to detect a 291-bp fragment derived from an allele(s) with Exon 1 deleted. The PCR conditions used were same as described above for routine genotyping.

Reverse Transcription/Quantitative-PCR (RT/qPCR)—Efnb1, Efnb2, T-bet, and RORγt mRNA levels were measured by RT/qPCR. Total RNA from cells was extracted using TRIzol® (Invitrogen) and then reverse-transcribed with Superscript II<sup>TM</sup> reverse transcriptase (Invitrogen). Primers used are listed in supplemental Table IA. qPCR conditions for the reactions was 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 or GAPDH mRNA levels were used as internal controls, and data were expressed as signal ratios of test gene mRNA/control gene mRNA.

Flow Cytometry—Single cell suspensions from the thymus, spleen, or lymph nodes were prepared and stained for flow cytometry as described in our previous publications (25–29). For intracellular antigen staining, cells were first stained with Abs against cell surface antigens, fixed with Cytofix/CytorpermTM solution (BD Biosciences), and then stained with mAbs against intracellular antigens. Antibodies for flow cytometry are shown in supplemental Table IB.

The following synthetic peptides were purchased from Sigma Genosys: gp(33–41), KAVYNFATC (LCMV-GP, H-2D<sup>b</sup>); np(396–404), FQPQNGQFI (LCMV-NP, H-2D<sup>b</sup>); gp(276–286), SGVENPGGTYC (LCMV-GP, H-2D<sup>b</sup>); and gp(61–80), GLNGPDIYKVQKFSVEFD (LCMV-GP, I<sup>A</sup>). PE-pp(33–41), PE-np(396–404), and PE-gp(276–286) H-2D<sup>b</sup> tetrameric complexes were synthesized in-house and used at 1:100 dilution as described previously (30). These MHC tetramers were used to detect LCMV-specific CD8<sup>+</sup> T cells. Briefly, splenocytes were first stained with PE-gp(33–41), PE-np(396–404), or PE-gp(276–286) tetramers for 30 min at 37 °C, followed by staining with FITC rat anti-mouse CD8<sub>a</sub> and APC-rat anti-mouse CD62L mAbs at 4 °C for another 20 min. 7-Aminoactinomycin D was used for exclusion of dead cells. After washing, cells were fixed in 0.5% paraformaldehyde, and samples were analyzed by flow cytometry.

Assessment of Thymocyte and T Cell Proliferation—Mice were injected intraperitoneally with 1 mg of 5-bromo-2-deoxyuridine (BrdU) in 0.2 ml of PBS and sacrificed 90 min later for thymocyte analysis. Another group of mice was injected with BrdU daily for 4 days and then sacrificed for spleen T cell analysis. Thymocytes or spleen cells were then isolated and stained with Abs against cell surface markers. They were subsequently permeabilized and stained with FITC-conjugated anti-BrdU Ab using a BrdU flow kit (BD Biosciences) according to the manufacturer’s instructions.

Generation of Bone Marrow Chimeras—Eight- to 10-week-old C57BL/6(6/Cd45.2<sup>+</sup>) × C57Bl6.SJL(Cd45.1<sup>+</sup>) F1 mice were irradiated at 1100 rads. Twenty four hours later, they received 4 × 10<sup>6</sup> T cell-depleted bone marrow cells from C57Bl6.SJL and dKO mice in a 1:1 ratio. Efnb1<sup>LoxP/LoxP</sup>/Efnb2<sup>LoxP/LoxP</sup> mouse bone marrow was used as a control. Thymocytes and spleen cells of the recipients were analyzed by flow cytometry 8–10 weeks following the bone marrow transplantation (BMTx).

In Vitro Th1, Th2, Th17, and Treg Polarization—Naive CD4<sup>+</sup> T cells were isolated from pooled splenocytes and lymph node cells using the naive CD4<sup>+</sup> T cells isolation kit (R&D Systems). Purity of the naive CD4<sup>+</sup> cells was routinely greater than 95%. Purified naive T cells (0.25 × 10<sup>6</sup> cells/well) were mixed with T cell-depleted irradiated (3000 rads) C57Bl6/6 feeder splenocytes (1.25 × 10<sup>6</sup> cells/well) and cultured in 96-well plates under Th1, Th2, Th17, or Treg polarization conditions, as described in our earlier publication (31), and then analyzed by flow cytometry.
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LCMV Infection and Clearance—LCMV-WE was obtained from Dr. R. M. Zinkernagel (Zurich, Switzerland). Viral stock was propagated in vitro, and viral titers were determined by the focus-forming assay as described previously (32). Mice were infected by the i.v. route with 200 focus-forming units of LCMV-WE. Mice were sacrificed 8 days post-infection to collect spleens for primary immune response analysis or 1 month later for memory immune response analysis. To evaluate viral clearance, mice were bled at days 4, 5, 7, 9, 11, 14, 23, and 28 post-infection, and LCMV titers in whole blood were measured by the focus-forming assay.

Detection of LCMV-specific IFN-γ and TNF-α-producing T Cells—One million splenocytes from LCMV-infected mice were seeded in single wells of 96-well round-bottomed plates. Cells were maintained in 5% RPMI 1640 medium supplemented with 100 units/ml mrlL-2, 10 μg/ml brefeldin A, 10 μM gp(33–41) or gp(61–80) peptide. After 5 h of incubation at 37 °C, cells were stained with phycoerythrin-conjugated rat anti-mouse CD8α or CD4 mAbs and 7-aminocoumarin D. Cells were then fixed, permeabilized and stained with allophycocyanin-rat anti-mouse TNF-α and FITC-rat anti-mouse IFN-γ mAbs. Frequency of IFN-γ and TNF-α-secreting T cells was determined by flow cytometry.

51Cr Release Assay—Eight days following LCMV infection, splenocytes were tested for cytotoxic activity in a standard 51Cr release assay as described previously (32).

Immunoblotting—dKO or control spleen T cells were purified with the EasySep™ T cell enrichment kit from Stemcell Technologies (Vancouver, British Columbia, Canada). These cells as well as total thymocytes were reacted with or without IL-6 (50 ng/ml) at 37 °C for 5 min for the detection of STAT3 phosphorylation. The cells were 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 inhibitors and phosphatase inhibitors (Complete™ protease inhibitor mixture and PhosSTOP phosphatase inhibitor mixture, Roche Diagnostics). The lysates were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes. STAT3 was detected with rabbit anti-mouse STAT3 antibody (Cell Signaling Technology) followed by HRP-conjugated donkey anti-rabbit IgG (GE Healthcare). The membranes were then stripped and reprobed with rabbit anti-mouse STAT3 mAb (clone 7D7, Cell Signaling Technology) for total STAT3 expression. The signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

RESULTS

Generation of Efnb1 and Efnb2 Conditional KO Mice—Efnb1 and Efnb2 null mutation caused embryonic lethality (4, 33). To study the function of these genes in the T cell compartment, we generated conditional KO mice with LoxP sites flanking Exon 1 of Efnb1 or Efnb2 (Fig. 1, A, B, E, and F). These mice (Efnb1f/f and Efnb2f/f) were crossed with transgenic mice expressing Cre recombinase driven by a proximal Lck promoter. As shown in the upper panels of Fig. 1, C and G, in the presence of the Lck-promoter Cre transgene, Exon 1 of Efnb1 or Efnb2 was deleted in thymocytes but was maintained in the genome of tail tissue in Lck-Cre-Efnb1f/f and Lck-Cre-Efnb2f/f mice.

RT-qPCR confirmed T cell-specific deletion of Efnb1 and Efnb2 mRNA in thymocytes and spleen T cells but not in spleen B cells (lower panels, Fig. 1, C and G). mRNA deletion in the thymocytes and spleen T cells was not complete in this quantitative assay, probably because of the less than perfect effectiveness of the LoxP-Cre deletion system. Abrogated cell surface expression of Efnb1 and Efnb2 proteins in thymocytes and peripheral T cells is demonstrated by flow cytometry (Fig. 1, D and H). The transgenic Cre in transgenic mice B6.Cg-Tg (Lck-Cre) 540Jxm1 generated by Dr. J. Martin was driven by the Lck proximal promoter (34). This proximal promoter becomes most effective in thymocytes starting from the DN3 stage (34, 35). Indeed, the effective deletion of Efnb1 started from the DN3 (double negative 3) stage and onward (Fig. 1D). For Efnb2, obvious deletion was observed from the DN4 stage and onward, although the deletion could also be seen at the DN2 and DN3 stage (Fig. 1H). Because the Lck proximal promoter ceases to be active after the SP stages (34, 35), the Efnb1 and Efnb2 deletion observed in peripheral T cells (Fig. 1, D and H) was caused by Efnb1 and Efnb2 deletion in their progenitor cells, i.e. DN3 and DN4 cells. Although the deletion in the DN3 and DN4 stage was not complete, the residual undeleted DN cells did not seem to develop into a major population in the periphery, and even the incomplete deletion was sufficient to cause phenotypic changes in the T cell compartment, as will be described below. It is worth mentioning that deletion of Efnb1 alone did not cause compensatory up-regulation of Efnb2 or vice versa (supplemental Fig. 1A).

Thymus and Spleen Features of dKO Mice—Lck-Cre-Efnb1f/f and Lck-Cre-Efnb2f/f single gene knock-out mice showed no discernable difference from the control Efnb1f/f and Efnb2f/f mice in terms of lymphoid organ size and histology (data not shown). We therefore generated Lck-Cre-Efnb1f/f/Efnb2f/f dKO mice. Efnb1f/f/Efnb2f/f mice were used as controls and are called WT controls in the subsequent text. The dKO mice had smaller thymi and spleens (Fig. 2A). The weight and cellularity of these organs and the T cell number in the spleen were diminished compared with those of WT control mice (Fig. 2B).

Efnb1 and Efnb2 Are Essential in Thymocyte Development—Thymocyte subpopulation analysis by flow cytometry of Lck-Cre-Efnb1f/f and Lck-Cre-Efnb2f/f single gene knock-out mice showed no significant abnormality (data not shown). The dKO thymi had smaller thymi and spleens (Fig. 2A). The weight and cellularity of these organs and the T cell number in the spleen were diminished compared with those of WT control mice (Fig. 2B).

Efnb1 and Efnb2 are essential for thymocyte development, as shown by the thymus size and T cell subset distribution in the dKO mice. However, the dKO mice had normal thymic weight and cellularity compared with WT controls, as shown in Fig. 2A. This result is consistent with the findings in previous studies (34, 35). Indeed, the effective deletion of Efnb1 started from the DN3 stage and onward, although the deletion could also be seen at the DN2 and DN3 stage (Fig. 1H). Because the Lck proximal promoter ceases to be active after the SP stages (34, 35), the Efnb1 and Efnb2 deletion observed in peripheral T cells (Fig. 1, D and H) was caused by Efnb1 and Efnb2 deletion in their progenitor cells, i.e. DN3 and DN4 cells. Although the deletion in the DN3 and DN4 stage was not complete, the residual undeleted DN cells did not seem to develop into a major population in the periphery, and even the incomplete deletion was sufficient to cause phenotypic changes in the T cell compartment, as will be described below. It is worth mentioning that deletion of Efnb1 alone did not cause compensatory up-regulation of Efnb2 or vice versa (supplemental Fig. 1A).

Thymic and splenic features of dKO mice were similar to those of control mice, as shown in Figure 2A. The weight and cellularity of these organs and the T cell number in the spleen were diminished compared with those of WT control mice (Fig. 2B).

Efnb1 and Efnb2 are essential in thymocyte development, as shown by the thymus size and T cell subset distribution in the dKO mice. However, the dKO mice had normal thymic weight and cellularity compared with WT controls, as shown in Fig. 2A.
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FIGURE 1—continued

G

H

Lck-Cre-Efnb2+/f

Efnb2+/f

Thymocytes

Relative expression of Efnb2

Thymocytes  T cells  B cells

Spleen
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The existence of different thymocyte subpopulations represents a balance between their proliferation, apoptosis, and progression. We wondered whether null mutation of both Efnb1 and Efnb2 affects the proliferation of thymocytes. The proliferation of DN3, DN4, DP, and SP subpopulations was assessed in vivo. As shown in Fig. 3C, no apparent difference in the proportion of BrdU-positive CD25+icTCRβ− or CD25+icTCRβ+ DN3 cells or CD25+icTCRβ− DN4 cells was observed between dKO and control thymi nor was there any difference at the DP, CD4SP, and CD8SP stages. We used icTCRβ− or icTCRβ+ to gate DN3 and DN4 subpopulations for their BrdU uptake (Fig. 3C). As dKO and control thymi did not differ significantly in their icTCRβ− cell percentage at the DN3 and DN4 stages (supplemental Fig. 1B), the BrdU results from Fig. 3C were not caused by abnormal icTCRβ expression in dKO cells. These results indicate that the reduced cellularity of certain subpopulations in the dKO thymus does not seem to be the result of their decreased proliferation.
We then evaluated whether thymocyte over-apoptosis was a factor causing the decrease of certain thymic subpopulations in dKO mice. Freshly isolated dKO thymocytes presented enhanced annexin V expression in all stages examined (DN3, DN4, DP, CD4SP, and CD8SP) compared with the controls (Fig. 3D). The higher apoptotic rate in DN3 cells does not seem to be consistent with increased DN3 cell percentage and absolute number in dKO thymi; it is possible that the effect of the blockage of progress from DN3 to DN4 resulted in an accumulation of DN3 cells that outweighed the detrimental effect of apoptosis in this subpopulation. The results of this section indicate that in the absence of Efnb1 and Efnb2, there is increased apoptosis in all subsets of thymocytes, and this could contribute to the reduced cellularity in the T cell compartment.

We assessed the CD3, CD5, CD24, and CD69 expression in dKO thymocyte subpopulations, particularly at the DP stage, where negative and positive selections occur. These cell surface proteins are critical in negative (CD3 and CD25) and positive (CD4 and CD8) selection processes. The expression levels of these proteins were analyzed by flow cytometry, and representative histograms are shown in Figure 3B.

FIGURE 3. Flow cytometry analysis of dKO thymocytes. A, DN, DP, CD4SP, and CD8SP subpopulations in dKO thymi. Thymocytes were analyzed for DN, DP, CD4SP, and CD8SP subpopulations. Representative histograms are shown, and the percentage of each subpopulation is indicated. Data from 20 to 27 pairs of dKO and control WT mice are summarized in Table 1. B, DN2, DN3, and DN4 subpopulations in dKO thymi. Thymocytes were first gated on Lin− cells, and the Lin− cells were analyzed for DN2, DN3, and DN4 subpopulation based on CD25 and CD44 expression. Representative histograms are shown. Data from 20 to 27 pairs of dKO and control WT mice are summarized in Table 2. C, DN3, DN4, DP, CD4SP, and CD8SP cell proliferation in dKO thymi according to BrdU staining. Control Efnb1+/Efnb2+/− and dKO mice were injected intraperitoneally with BrdU (1 mg/mouse), and their thymocytes were analyzed by flow cytometry 90 min later. Thymocytes were stained with lineage markers as well as anti-BrdU Ab. Percentages of BrdU-positive DN3 (CD25−icTCRβ− and CD25+icTCRβ−), DN4 (CD25+icTCRβ−), DP, CD4SP, and CD8SP cells are shown. D, dKO thymocyte apoptosis. dKO and WT control thymocytes were stained with annexin V and lineage markers ex vivo or cultured for 24 h in plain medium and then analyzed as indicated. The percentage of annexin V-positive cells in DN3, DN4, DP, CD4SP, and CD8SP cells is shown. In C and D, the experiments were repeated more than three times, and representative histograms are shown.
TABLE 1
Percentage and absolute numbers of CD4SP, CD8SP, DP, and DN subpopulations

|         | CD4SP |         | CD8SP |         | DP |         | DN |         |
|---------|-------|---------|-------|---------|----|---------|----|---------|
| % Cells | (×10⁶) | % Cells | (×10⁶) | % Cells | (×10⁶) | % Cells | (×10⁶) | % Cells | (×10⁶) |
| WT      | 10.60 ± 3.75 | 13.60 ± 4.82 | 4.25 ± 1.69 | 5.84 ± 2.61 | 79.31 ± 6.37 | 112.93 ± 38.34 | 5.42 ± 2.90 | 6.84 ± 2.85 |
| dKO     | 9.20 ± 3.82 | 6.93 ± 4.66 | 4.78 ± 1.82 | 3.91 ± 2.16 | 74.39 ± 6.33 | 61.43 ± 32.22 | 16.11 ± 4.21 | 7.98 ± 3.97 |

*p < 0.001.

| Table 2 |
|---------|

Percentage and absolute numbers of DN2, DN3, and DN4 thymocytes

|         | DN2 |         | DN3 |         | DN4 |         |
|---------|-----|---------|-----|---------|-----|---------|
| % Cells | (×10⁶) | % Cells | (×10⁶) | % Cells | (×10⁶) | % Cells | (×10⁶) |
| WT      | 3.03 ± 2.015 | 0.30 ± 0.14 | 37.70 ± 11.02 | 4.25 ± 2.22 | 53.22 ± 15.75 | 5.52 ± 3.06 |
| dKO     | 2.97 ± 1.42 | 0.37 ± 0.23 | 54.16 ± 12.63 | 6.33 ± 2.84 | 40.12 ± 13.76 | 4.44 ± 2.59 |

*p < 0.001.

Are there additional factors causing the skew of memory versus naive T cell ratio in dKO mice? We assessed the apoptosis of these cells from the spleen. Both naive and memory type CD4 and CD8 cells from dKO mice showed an increased annexin V-positive percentage compared with those from WT mice (Fig. 4E), but naive type cells did not present a higher proportion compared with memory type cells. This higher apoptotic rate of all the dKO subsets likely contributes to the reduced peripheral T cell populations in dKO mice, but it is not a reason for a relatively higher percentage of memory type T cells in these mice. We also demonstrated that both naive and memory type T cells from dKO mice manifested a similar BrdU uptake in vivo compared with cells from WT mice (supplemental Fig. 3A). Taken together, these results suggest that the significant reduction of naive dKO T cells compared with memory dKO T cells is due to reduced output of naive T cells from the dKO thymus, but Efnb1 and Efnb2 do not seem to affect significantly the conversion from naive to memory type T cells.

We next assessed different functional T cell subpopulations either ex vivo or following in vitro differentiation. There was no change in the relative proportion of CD25⁺FoxP3⁺ Treg cells in the dKO spleen and lymph nodes relative to the control spleen and lymph nodes when examined ex vivo (supplemental Fig. 3B). When naive CD4 cells were cultured under Th1, Th2, Th17, and Treg conditions (Fig. 4F), the dKO cells (lower row) showed compromised differentiation toward Th1 (IFN-γ IL-17 cells; 1st panel) and Th17 (IFN-γ IL-17⁺ cells; 3rd panel) but not Th2 (IL-4⁺ IFN-γ⁻ cells; 2nd panel) nor Treg...
**FIGURE 4. Flow cytometry analysis of dKO peripheral lymphocytes and their in vitro differentiation.** A, T and B cell subpopulations in dKO spleens. Spleen and lymph node cells were stained with anti-CD3 and anti-CD20 Abs for T and B cells, respectively. Percentage of CD3- and B220-positive cells is shown. B, CD4 and CD8 T cell subpopulations in dKO spleens. Spleen or lymph node cells were stained with anti-CD4 and anti-CD8 Abs for CD4 and CD8 T cells, respectively. Percentage of CD4- and CD8-positive cells is shown. C, naive and memory T cell populations in dKO spleens. CD44loCD62Lhi and CD44hiCD62Llo cells among CD4 and CD8 splenocytes were analyzed, and their percentage is shown in the histogram. The bar graph at left illustrates the absolute number of CD44loCD62Lhi and CD44hiCD62Llo cells in the spleen based on the calculation of the percentage and spleen cellularity of 10 pairs of dKO and WT mice. ***, p < 0.001 (Student’s t tests). D, CD44loCD62Lhi and CD44hiCD62Llo cells in the dKO SP thymocytes. CD4SP and CD8SP thymocytes were gated, and CD44loCD62Llo cells among these subpopulations were analyzed by flow cytometry. Their percentage is shown in the histograms. E, apoptosis of spleen CD44loCD62Lhi and CD44hiCD62Llo T cells of dKO mice. dKO and WT spleen CD44loCD62Lhi and CD44hiCD62Llo cells in the CD4 and CD8 subpopulations were analyzed ex vivo for their annexin V expression by flow cytometry. F, in vitro Th1, Th2, Th17, and Treg differentiation according to intracellular IFN-γ, IL-4, IL-17, and Foxp3 staining. Naive CD4 cells were cultured under Th1, Th2, Th17, or Treg conditions (3 days for Th1, Th17, and Treg and 5 days for Th2). Cells were further stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 h before being harvested. The intracellular expression of IFN-γ, IL-4, IL-17, and Foxp3 was determined by flow cytometry. Percentage of IFN-γ, IL-4, IL-17, and Foxp3-positive cells is shown. G, expression of T-bet and RORγt mRNA in Th1 and Th17 cells. The T-bet and RORγt mRNA from in vitro differentiated Th1 and Th17 cells, as described in F, was quantified by RT-qPCR, using GAPDH as an internal control. Data from five independent experiments are expressed as the means ± S.D. of the ratios of T-bet/GAPDH or RORγt/GAPDH signals. *, p < 0.05 (Student’s t test). Experiments in this figure were repeated more than three times, unless indicated otherwise, and representative data are shown.
In testing the in vitro Th1 and Th17 differentiation, we started with the same number of naive CD4 cells. The compromised Th1 and Th17 development of dKO CD4 could be due to defective expansion or differentiation, or both. To distinguish these possibilities, we examined the expression of transcription factors T-bet and RORγt, which are essential transcription factors in Th1 and Th17 cell differentiation, respectively. As shown in Fig. 4G, under Th1 culture conditions, there was a small but significant increase in T-bet expression in dKO T cells compared with WT T cells, although the RORγt expression of the dKO and WT cells under Th17 culture conditions was similar. The reason for this small increase is not clear, but this result suggests that the defective Th1 and Th17 cell development of dKO CD4 T cells is probably mainly due to compromised expansion rather than differentiation.

**Pivotal Role of Efnb1 and Efnb2 Reverse Signaling in T Cell Development and Homeostatic Expansion**—Ephs are capable of transducing signals in both directions (2, 3). We wanted to understand which direction of signaling was critical for the observed phenotype in the dKO mice. To this end, we used a model of whole body irradiation followed by BMTx, using B6.SJL bone marrow cells to compete with dKO bone marrow cells. In this model, CD45.1 single positive cells were derived from competitor B6.SJL bone marrow cells; CD45.2 single positive cells were derived from the dKO bone marrow cells; and CD45.1/CD45.2 double positive cells were derived from residual recipient bone marrow cells and peripheral cells.
As shown in Fig. 5A, 63.9% of the thymocytes in the control recipients were derived from WT donor bone marrow cells, whereas in the test recipients only 0.4% thymocytes were derived from dKO donor bone marrow cells (CD45.2 single positive cells; upper left panel). Similar significant differences were also found in the spleen (Fig. 5A, 2nd row), lymph nodes (3rd row), and blood (last row). This clearly indicates that the dKO bone marrow cells were significantly inferior to the WT control bone marrow cells in their capacity to compete with B6.SJL bone marrow cells to develop and expand in the void niche created by irradiation.

As expected, the proportion of CD3$^+$ T cells in the spleen, lymph nodes, and blood derived from dKO bone marrow cells was significantly lower than the proportion derived from control bone marrow cells (Fig. 5B). However, the percentage of B

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**FIGURE 5.** Compromised development and expansion of dKO bone marrow cells and mature T cells in irradiated recipients. A, dKO bone marrow cells failed to compete with WT bone marrow cells in irradiated recipients. T cell-depleted dKO and WT bone marrow cells (CD45.2$^+$) 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 × B6.SJL F1 recipients. After 90 days, cells from the thymus, spleen, lymph node, and blood were analyzed for CD45.2 and CD45.1 staining. Percentages of CD45.2$^+$ cells (derived from dKO and WT control bone marrow cells), CD45.1$^+$ cells (derived from competing B6.SJL bone marrow cells), and CD45.1$^+$/CD45.2$^+$ cells (derived from residual cells of the recipients) are shown. B, dKO bone marrow cells in the presence of competing B6.SJL bone marrow cells had significantly reduced capability to develop into T cells in the periphery. In the whole body irradiation-BMTx model described in A, B220$^+$ B cells and CD3$^+$ T cells among CD45.2$^+$ cells (derived from dKO or control WT bone marrow cells) in the spleen, lymph node, and blood were determined by flow cytometry 90 days later; percentages are shown. C, dKO T cells presented failed homeostatic expansion in sublethally irradiated recipients. B6.SJL mice (CD45.1$^+$) were sublethally irradiated at 600 rads and transplanted i.v. with 4 × 10$^6$ CFSE-labeled spleen cells from dKO or WT control mice (CD45.2$^+$). The histograms show profiles of carboxyfluorescein succinimidyl ester (CFSE)-positive cells gated on CD4$^+$ CD45.2$^+$ and CD8$^+$ CD45.2$^+$ cells. Experiments in this figure were repeated more than three times, and representative histograms are shown.
cells (B220-positive cells) derived from dKO bone marrow cells was greater than that derived from control bone marrow, indicating a T cell-specific development defect due to the Lck-Cre-caused deletion of both Enb1 and Enb2. In this system, Ephs of dKO thymocytes and peripheral T cells should have sufficient forward Efnb1 and Efnb2 stimulation from competing WT B6.SJL bone marrow-derived cells, whose Efnb1 and Efnb2 expression was normal. Therefore, we could attribute the defective development and expansion of dKO thymocytes mainly to the absence of both Efnb1 and Efnb2 reverse signaling.

To understand whether mature dKO T cells were incompetent in homeostatic expansion, we transferred CFSE-labeled dKO or WT spleen cells into sublethally irradiated B6.SJL mice. As shown in Fig. 5C, dKO donor T cells showed significantly lower homeostatic proliferation in vivo compared with WT control donor T cells. This compromised homeostatic proliferation of mature dKO T cells along with their increased apoptosis and reduced thymocyte output as described above are likely all contributing factors resulting in the significant decrease of peripheral T cells in the dKO mice.

Efnb1 and Efnb2 Deletion in the T Cell Compartment Results in Compromised Immune Responses to LCMV Infection—The reduced peripheral T cell population and defective T helper cell differentiation in dKO mice strongly suggested that these mice might have incompetent immune responses in vivo. We therefore assessed their anti-LCMV immune response. Eight days after the mice were infected with LCMV (strain WE), there was a significant increase in the absolute number of total spleen cells in both dKO and control mice (Figs. 6A versus 2B). The
increase of CD8 cells surpassed that of CD4 cells, and they became the dominant cell population in the spleen (Fig. 6A), because the antiviral response is mainly a CD8 cell-mediated event. However, the absolute cell number of both CD4 and CD8 cell populations was significantly lower in dKO mice compared with that of the control Efnb1<sup>−/−</sup>/Efnb2<sup>−/−</sup> mice. Using LCMV antigen-specific tetramers, it was shown that the percentage of gp33<sup>−</sup>, np396<sup>−</sup>, and gp276-specific CD8 cells among the total CD8 cells was comparable in both dKO and control mice (Fig. 6B), suggesting a similar rate of clonal expansion following viral antigen stimulation. The absolute number of tetramer-positive CD8 cells per spleen tended to be lower in dKO mice (Fig. 6C), probably reflecting a smaller starting T cell population, but they did not reach statistical significance except the gp33-positive cells at the present sample size, likely due to the level of variation.

We next examined the presence of antigen-specific cytokine-producing spleen T cells in the virus-infected mice. As shown in Fig. 6D (upper row), the relative percentage of antigen-specific IFN-γ-producing CD8 cells, but not CD4 cells, was significantly lower in dKO than control spleens 8 days post-infection (1st panel, upper row). This was also the case for antigen-specific TNF-α-producing CD8 cells (Fig. 6D, 2nd panel, upper row). Such reduction is probably caused mainly by the reduced number of antigen-specific CD8 cells to start with, but their compromised differentiation might also play a role. The defective dKO CD4 cell help, as demonstrated in our in vitro study in Fig. 4F, probably also contributed to the compromised CD8 cell development. Antigen-specific IFN-γ and TNF-α secreting CD4 cells in the dKO spleen were lower in percentage and number compared with those in the control spleen, although the difference was not statistically significant at the current sample size (n = 4; Fig. 6D, upper row).

Interestingly, we noticed that 32 days following virus infection, the dKO mice caught up with the control mice in that that the percentage and number of virus antigen-specific IFN-γ and TNF-α-producing CD4 and CD8 cells became similar to the latter (Fig. 6D, lower row). On day 32 post-virus infection, the tetramer-positive cells were of CD4<sup>+</sup> memory T cell type (data not shown), and this is consistent with our earlier findings that memory T cell development in dKO mice seems unimpaired (Fig. 4C). On day 8 post-infection, virus-specific cytotoxic T cell activity among total spleen cells from the dKO mice was significantly lower than that of control mice (Fig. 6E). However, on a per cell basis, the cytotoxic T cell activity was similar between the dKO and controls (data not shown), suggesting that the reduced cytotoxic T cell activity among spleen cells was mainly due to reduced number of virus-specific CD8 cells (Fig. 6C) in the dKO spleen. LCMV virus clearance was monitored at different days following virus infection. The virus was detected in blood 7 days after infection but was cleared at 14 days in both dKO and control mice (Table 3).

Our additional results indicate that the dKO mice also had compromised cardiac allograft rejection response (supplemental Fig. 3C). Results from the virus infection along with allograft rejection show that Efnb1 and Efnb2 are relevant in T cell immune response in vivo. It is to be noted that the heavy redundancy in the immune system and the remaining T cell capacity, some of which might depend on other Eph/Efn molecules and some of which might be from T cells escaped from the incomplete Lck-Cre-mediated deletion, are sufficient to clear the LCMV infection eventually.

### DISCUSSION

We have shown that Efnb1 and Efnb2 have critical functions in thymocyte development, as well as peripheral T cell function. They are also involved in the antiviral immune responses in vivo. We present evidence that Efnb1 and Efnb2 are necessary for T cell development.
for proper IL-6 signaling, which is essential in the observed dKO phenotype.

Because of the promiscuous interaction mostly among Ephs and Efnbs, different Ephs and Efnbs have overlapping functions; as a result, deletion of only one of them might not always reveal discernable phenotypes in the immune system. Indeed, our previous study showed that although Ephb6 null mutation in mice resulted in compromised peripheral T cell function, the animals had no obvious defect in thymus structure or thymocyte development (29). We also examined thymus size, structure, and cellularity of Lck-Cre-Efnb1f/f and Lck-Cre-Efnb2f/f mice but found no drastic defects (data not shown). When both Efnb1 and Efnb2 in the T cell compartment were null mutated, a significant reduction in thymus size and cellularity was observed. This clearly indicates that Efnb1 and Efnb2 have overlapping functions and could complement each other if one is missing. It is to be noted that deletion of Efnb1 or Efnb2 alone did not induce compensative up-regulation of the other (data not shown).

The Cre recombinase driven by the proximal Lck promoter becomes most active at the DN3 and DN4 stage, and the promoter is no longer active after the SP stage (34, 35). However, the Efnb1 and Efnb2 deletion in the DN3 and DN4 stages led to their effective deletion in subsequent offspring T cell populations in the dKO thymus and spleen. Although the deletion in the DN3 and DN4 stages was less than complete, the cells that escaped the deletion never became a significant population in the subsequent stages. The functional defects detected in the peripheral T cells in terms of cytokine production, T cell homeostatic expansion, antiviral responses, and IL-6R signaling confirm that most of the T cells are not escapees of undeleted cells from the thymus, and our model is sufficient and useful to elucidate the role of these two molecules in the T cell compartment.

In some biological systems, Cre expression might interfere with function of certain cell types, but in our system this is not an issue, as our Efnb1f/f and Efnb2f/f mice with Lck-driven Cre expression manifested no abnormalities in terms of thy-
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mocyte and T cell subpopulations and functions (data not shown).

Efnb1 and Efnb2 are capable of both forward signaling by stimulating Ephps, mostly Ephb members, and reverse signaling by accepting Ephb stimulation. In the dKO thymus, the stroma cells still have normal Efnb1 and Efnb2 expression and can provide forward signaling to the developing dKO thymocytes through their Ephbs, but obviously such forward signaling is not sufficient. Consequently, the compromised dKO thymocyte development is an indication that reverse signaling is important to this process. The thymus is packed with thymocytes, and most contacts that a thymocyte receives are from fellow thymocytes. As a result, one could argue that in the dKO thymus, thymocyte Ephbs might not receive enough forward signaling from neighboring thymocytes that lack Efnb1 and Efnb2. To address this concern, dKO bone marrow cells were cotransplanted with wild type B6.SJL bone marrow cells at a 1:1 ratio into irradiated recipients (C57BL/6×B6.SJL F1). In this system, the competitor B6.SJL bone marrow cells as well as recipient thymic stroma cells had normal Efnb1 and Efnb2 expression. The dKO thymocytes should have had sufficient forward signaling from the Efnb1 and Efnb2 of neighboring competing B6.SJL-derived thymocytes as well as from C57BL/6×B6.SJL F1 stroma cells. However, they failed to compete with the B6.SJL-derived bone marrow cells; consequently, there was a significant decrease in the number of thymocytes and peripheral T cells. This indicates that Efnb1 and Efnb2 reverse signaling but not forward signaling is largely responsible for the defect observed in dKO mice in terms of reduction of various thymocyte subpopulations and peripheral T cells.

Such reverse signaling was also critical in mature T cell function. Naive dKO CD4 cells in the periphery were defective in their ability to differentiate into Th1 and Th17. Under Th1 and Th17 culture conditions, Efnb1- and Efnb2-expressing WT feeder cells were present, so the dKO naive CD4 T cells were not lacking forward signaling, especially when considering that the ratio of the feeder cells to naive T cells was 5:1. Thus, the defective Th1 and Th17 development in vitro seen in this study is also mainly the consequence of a lack of reverse signaling through Efnb1 and Efnb2.

Although we showed with the above-described two experimental models that reverse signaling from Eph through Efnb1/ Efnb2 is essential for thymocyte development and peripheral T cell function, forward signaling from Efnb1 and Efnb2 through Eph could still play a role, maybe a minor one, in promoting thymocyte survival and T cell function. This is evidenced in our previous study in that forward signaling of Efnb1 could enhance thymocyte survival (45), and Efnb1 and Efnb2 could costimulate T cells and enhance lymphokine production by forward signaling (25, 27).

We have shown that in Efnb1 and Efnb2 dKO mice, the peripheral naive T cell population was more affected than memory T cell population in terms of absolute cell number as well as percentage. We also found that the population of antigen-specific IFNγ- and TNFα-secreting memory type CD8 cells in LCMV-infected dKO and WT mice on day 32 post-infection was similar (data not shown), although initially on day 8 post-infection, dKO mice showed reduced antigen-specific IFNγ- and TNFα-secreting CD8 cells (Fig. 6D). This suggests again that Efnb1 and Efnb2 affected less the memory type T cells in terms of function. Additional studies on secondary immune response in dKO mice to assess the function of both CD4 and CD8 memory T cells are warranted to confirm such suggestive indications. Overall, our data are consistent with the report by Almeida et al. (46) in that reduced thymic output of T cells preferentially affects the naive T cell pool but exerts less effect on the memory T cells, probably because of a better compensatory mechanism than the latter.

We observed that the reduction of peripheral dKO T cells in the whole body irradiation-BMTx model was far more significant than in dKO mice. This suggests that dKO thymocytes and T cells are significantly inferior to their wild type competitors in their development in the thymus and subsequently their peripheral homeostatic expansion. Such inferiority was less obvious when dKO T cell progenitors were allowed to develop without competitors in the thymus and periphery in the dKO mice, because they could take all the time and space to develop at their own pace.

In thymocytes and peripheral T cell compartment, deletion of Efnb1 and Efnb2 led to increased cell death according to ex vivo annexin V staining, but this minimally affected their proliferation based on BrdU uptake. However, when dKO T cells were transplanted into irradiated B6.SJL recipients, their homeostatic proliferation was significantly lower than WT control T cells. The T cell homeostatic expansion in irradiated mice is much faster than in mice without irradiation. Efnb1 and Efnb2 probably play a more critical role during the fast expansion, either by their direct effect or indirectly via modulating cytokine signaling pathways as will be discussed below.

Efnbs have short intracellular tails, which do not possess any enzymatic activities but have six tyrosine residues. Some of the tyrosine residues are rapidly phosphorylated upon engagement of Ephps (47). The phosphorylated tyrosines then bind to adaptor proteins containing Src homology 2 domains such as Grab4 (48) and Disheveled (49). These proteins in turn interact with different signaling pathways and transduce signals to achieve biological effects on the cells. Near the C terminus of Efnb1, there is a conserved YXXQ motif. Recently, Bong et al. (50) reported that once the tyrosine in this motif is phosphorylated, it is capable of binding the Src homology 2 domain-containing protein STAT3. Such association leads to STAT3 phosphorylation and activation in a JAK2-dependent manner; JAK2 can be found in the Efnb1-STAT3 complex. It is to be noted that the intracellular tail of Efnb2 also contains the YXXQ motif, although Efnb2 is less potent than Efnb1 in leading to STAT3 phosphorylation (49). STAT3 is in the IL-6 signaling pathway, as gp130, the signaling subunit of IL-6R complex, contains the YXXQ motif, which binds to STAT3. The binding of IL-6 to IL-6Rα results in the formation of a hexamer containing two molecules of IL-6, two molecules of IL-6Rα, and two molecules of gp130. This complex activates JAK, which in turn phosphorylates gp130-associated STAT3 and leads to STAT3 activation (51). Our data demonstrate that in the absence of Efnb1 and Efnb2, IL-6-induced STAT3 phosphorylation was significantly dampened. This suggests that Efnb1 and Efnb2 act in an additive or synergistic fashion to enhance IL-6 signaling through
STAT3 phosphorylation; in other words, the full STAT3 activation, and hence the full-strength IL-6R activation, requires the participation of both IL-6R signaling and Efnb1/Efnb2 reverse signaling.

Indeed, many phenotypes observed in the dKO mice could be attributed, at least to some extent, to reduced IL-6 signaling in the T cell compartment. We observed reduced thymocyte and peripheral T cell numbers in dKO mice, and this is also the case in IL-6 KO mice (42). The dKO CD4 cells presented diminished Th17 but not Treg differentiation in vitro; the former but not the latter requires the presence of exogenous IL-6 in the culture system (43). The dKO T cells had compromised homeostatic expansion; in agreement with this finding, Zasragoza et al. (52) showed that IL-6 is required for the survival of naive T cells during such expansion. Our dKO manifested compromised antiviral immunity against LCMV; similarly, IL-6 KO mice show defective immune responses against vesicular stomatitis and vaccinia viruses (42); recently, Pellegrini et al. (44) have demonstrated that IL-7 can enhance immunity against chronic LCMV infection and that an important part of this effect is via IL-6 signaling.

It is prudent to say that it is entirely possible that the mechanisms of action of Efnb1 and Efnb2 in the T cell compartment are not restricted to dampening the IL-6 signaling via direct acting of Efnb1 and Efnb2 on STAT3 phosphorylation. As discussed above, forward signaling from Efnb1 and Efnb2 on T cells to neighboring T cells or other immune-related cells likely contributes to the integrity of an immune response. Efnbs on T cells might also affect the function of other signaling pathways by associating with other adaptor proteins in addition to STAT3. Efnbs are also known to bind to other cell surface molecules (53), and such binding can influence their functions of those molecules.

Although most documented functions of Ephps and Efns are related to pattern formation, the defective T cell differentiation and function in the dKO mice obviously have little to do with such classical functions of Ephp and Efns. This indicates that Efnb1 and Efnb2 have previously underappreciated functions that go beyond their classical duty.

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