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Role of EFNBs and EphB4 in T cell development and functions

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Résumé

Kinases Eph est la plus grande famille de tyrosines kinases récepteurs Éphrines (EFN) est un ligand de Ephs. Eph et EFN sont toutes les molécules de surface cellulaire. L'interaction entre Ephs et EFNs permet de transmettre des signaux dans les deux directions (c.-à-d. partir de Ephs à EFNs, et de EFNs à Ephs.)

Eph et EFNs sont largement impliqués dans divers processus développementaux, physiologiques et physiopathologiques. Notre groupe et d'autres groupes ont rapporté les rôles de Ephs / EFNs dans le système immunitaire. Pour approfondir la fonction de EphBs / EFNBs dans le développement des lymphocytes T et des réponses immunitaires, nous avons généré des souris EFNB1, EFNB2, et EphB4 knock-out conditionnel (KO) et des souris EFNB1 / 2 doubles KO.

Dans les projets qui utilisent EFNB1 et EFNB2 comme souris knock-out, nous avons spécifiquement supprimé EFNB1 ou EFNB2 dans les cellules T. Les souris présentaient une taille normale, la cellularité du thymus et de la rate, ainsi que des sous-populations de cellules T étaient normales dans ces organes. Les progéniteurs de la moelle osseuse de souris KO et les souris WT ont repeuplé les organes lymphoïdes de l'hôte à des degrés similaires. L'activation et la prolifération des cellules KO T étaient comparables à celles des souris témoins. Les cellules CD4 naïves KO différenciées en Th1, Th2, Th17 et Treg étaient similaires aux cellules CD4 naïves de souris contrôle. Chez les souris KO EFNB2, nous avons observé une augmentation relative importante des thymocytes CD4CD8 : les double négatifs dans le thymus. L'analyse par cytométrie en flux a révélé qu'il y avait une augmentation modérée de la sous-population DN3 dans le thymus. Les résultats suggèrent qu'EFNB2 est impliqué dans le développement des thymocytes. Nos résultats indiquent que les fonctions de EFNB1 et EFNB2 dans le compartiment des cellules T pourraient être compensées entre eux ou par d'autres EFNB. La redondance des fonctions suggèrent le contrôle critique d'EFNB1 et EFNB2 dans le développement des cellules T.

Dans le projet, en utilisant EFNB1/B2 (modèle double KO) (dKO), nous avons observé une fonction de régulation de EFNB1 et EFNB2. dans la stabilisation de l'expression l'IL-7R α , à la surface des cellules T, IL-7 joue un rôle important dans le développement des thymocytes, l'homéostasie des lymphocytes T , et leur survie. IL-7R α subit une internalisation

contraignante de IL-7. Chez les souris DKO, nous avons observé une perte d'expression de l' IL-7Rα dans les thymocytes et les cellules T. En outre, l' internalisation IL-7Rα a été accélérée dans les cellules CD4 dKO, suite à la stimulation IL-7. Dans la lignée cellulaire de lymphome T, EL4, la surexpression de EFNB1 ou EFNB2 retarde l'internalisation de l'IL-7Rα. Nous avons aussi démontré les signalisations compromises de l' IL-7 et de la prolifération homéostatique des cellules T dKO. Les études du méchanisme qui utilisent la fluorescence de transfert d'énergie par résonance et immunoprécipitation ont montré que l'interaction physique de EFNB1 et EFNB2 avec IL-7R était probablement responsable du retard de l' internalisation IL-7Rα.

Dans le dernier projet, nous avons étudié le développement des cellules T et la fonction des cellules épithéliales médullaires du thymus (mTEC), chez les souris knock-out EphB4. Les souris KO EphB4 ont démontré un poids et une cellularité qui sont normaux. La fonction et le développement de cellules T ne sont pas influencés par la suppression de l' EphB4. Enfin, les souris KO ont développé une hypersensibilité de type retardée normale.

Dans l'ensemble, nos résultats suggèrent que l'interaction globale de croisement entre Eph et les membres de la famille EFN pourrir compenser la fonction d'un membre supprimé. Seule la suppression simultanée de plusieurs EFNBs va révéler leur vraie fonction dans le système immunitaire. En fait, une telle redondance montre les rôles vitaux d'Ephs et EFNS dans le système immunitaire.

Mots-clés : Eph; EFNB; KO, la délétion génique conditionnelle; le développementdes cellules T, la fonction des cellules T; IL-7Rα; cellules épithéliales Thymique médullaire (mTEC).

Abstract

Eph kinases are the largest family of cell surface receptor tyrosine kinases. The ligands of Ephs, ephrins (EFNs), are also cell surface molecules. Ephs interact with EFNs and the receptors and ligands transmit signals in both directions, i.e., from Ephs to EFNs and from EFNs to Ephs.

Ephs and EFNs are widely involved in various developmental, physiological pathophysiological processes. Our group and others have reported the roles of Ephs/EFNs in the immune system. To further investigate the function of EphBs/EFNBs in T cell development and responses, we generated EFNB1, EFNB2, EphB4 conditional gene knockout (KO) mice and EFNB1/2 double KO mice.

In the projects using EFNB1 and EFNB2 knockout mice, we specifically deleted EFNB1 or EFNB2 in T cells. The mice had normal size and cellularity of the thymus and spleen as well as normal T cell subpopulations in these organs. The bone marrow progenitors from KO mice and WT mice repopulated the host lymphoid organs to similar extents. The activation and proliferation of KO T cells was comparable to that of control mice. Naïve KO CD4 cells differentiated into Th1, Th2, Th17 and Treg cells similar to naïve control CD4 cells. In EFNB2 KO mice, we observed a significant relative increase of CD4CD8 double negative thymocytes in the thymus. Flowcytometry analysis revealed that there was a moderate increase in the DN3 subpopulation in the thymus. This suggests that EFNB2 is involved in thymocyte development. Our results indicate that the functions of EFNB1 and EFNB2 in the T cell compartment could be compensated by each other or by other members of the EFN family, and that such redundancy safeguards the pivotal roles of EFNB1 and EFNB2 in T cell development and function.

In the project using EFNB1/B2 double knockout (dKO) model, we revealed a novel regulatory function of EFNb1 and EFNb2 in stabilizing IL-7Rα expression on the T cell surface. IL-7 plays important roles in thymocyte development, T cell homeostasis and survival. IL-7Rα undergoes internalization upon IL-7 binding. In the dKO mice, we observed reduced IL-7Rα expression in thymocytes and T cells. Moreover, the IL-7Rα internalization was accelerated in dKO CD4 cells upon IL-7 stimulation. In T cell lymphoma cell line, EL4, over-expression of either EFNB1 or EFNB2 retarded the internalization of IL-7Rα. We further demonstrated

compromised IL-7 signaling and homeostatic proliferation of dKO T cells. Mechanism study using fluorescence resonance energy transfer and immunoprecipitation demonstrated that physical interaction of EFNB1 and EFNB2 with IL-7Rα was likely responsible for the retarded IL-7Rα internalization.

In the last project, using medullary thymic epithelial cell (mTEC)-specific EphB4 knockout mice, we investigated T cell development and function after EphB4 deletion in mTEC. EphB4 KO mice demonstrated normal thymic weight and cellularity. T cell development and function were not influenced by the EphB4 deletion. Lastly, the KO mice developed normal delayed type hypersensitivity.

Overall, our results suggest that comprehensive cross interaction between Eph and EFN family members could compensate function of a given deleted member in the T cell development, and only simultaneous deletion of multiple EFNBs will reveal their true function in the immune system. In fact, such redundancy signifies vital roles of Ephs and EFNs in the immune system.

Keywords : Eph; EFNB; conditional gene knockout; T cell development; T cell function; IL-7Rα; Thymic epithelial cells

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List of abbreviations

Abi-1 Abl-interacting protein-1

AHR airway hyperreactivity

AIRE autoimmune regulator

APC antigen-presenting cell

APECED autoimmune polyendocrinopathy-candidiasis-ectodermaldystrophy

BAL bronchoalveolar lavage

CAP Cbl-associated protein

CIA collagen-induced arthritis

CMJ cortico–medullary junction

CNS central nerve system

CRD cysteine-rich region

cTEC cortex thymic epithelial cells

DC dendritic cell

DIX Dishevelled-Axin

DN double negative

DP double positive

Dsh Dishevelled

DTH delayed type of hypersensitivity

EAE experimental autoimmune encephalomyelitis

EC endothelial cell

Efn Ephrins

Eph Erythropoietin-producing hepatocyte kinase

ephexin1 Eph-interacting exchange protein 1

ETP early thymic progenitor

FGFR fibroblast growth factor receptor

FNIII fibronectin type III

FRET Fluorescence resonance energy transfer

Frz Frizzled

GAP GTPase-activating protein

GEF guanine nucleotide exchange factor

GPI glycosylphosphatidylinositol;

GRIP1 Glutamate receptor interacting protein

HCV hepatitis C virus

HIF-1 hypoxia-inducible factor-1

HIV Human immunodeficiency virus

HMG high mobility group

HSC hematopoietic stem cells

IBD inflammatory bowel diseases

IL interleukin

Ip Immunoprecipitation

IPEX Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

iTreg induced regulatory T cells

JAK Janus Kinase

JM juxtamembrane

JNK Jun kinase

K5 Keratin-5

LBD ligand binding globular domain

MAP mitogen-activated protein;

MAPK mitogen-activated protein kinase

MBD2 methyl-CpG-binding domain protein 2

MHC major histocompatibility complex

MS multiple sclerosis

mTEC medullar thymic epithelial cells

nTreg natrual regulatory T cell

NURD nucleosome remodelling and histone deacetylase

PAK p2l-activated kinase

PDZ Psd-95, Dlg and ZO1

PH pleckstrin homology

Pl3K phosphatidylinositol-3-kinase

PTB phosphotyrosine-binding

PTH parathyroid hormone

PTHrP parathyroid hormone-related protein

PTP-BL protein tyrosine phosphatase BAS-like

Pyk2 proline-rich tyrosine kinase 2

RA Rheumatoid arthritis

RBD receptor-binding domain

RORgt retinoid orphan nuclear receptor

RTK receptor tyrosine kinase;

SAM sterile alpha motif;

SDF-1 stromal-cell-derived factor 1

SH2 Src homology 2

SOS son of sevenless

SP single positive

STAT Signal transducer and activator of transcription

T-bet T-box expressed in T cell

TM transmembrane

TNF tumour-necrosis factor

TSLP thymic stromal lymphopoietin

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I. Introduction

I.1 Eph and Ephrin

Erythropoietin-producing hepatocyte kinases (Ephs) are the largest family of receptor tyrosine kinases (RTK). Upon interaction with their ligands the Ephrins (EFN), downstream signaling cascades of both receptor and ligand are initiated which is referred as bidirectional signaling [1]. Eph and EFN play important roles in a wide range of biological processes from neuron axon guidance, angiogenesis, skeletal development, tissue patterning to immune responses [2, 3].

I.1.1 Classification and structure of Eph and EFN

The Eph family includes fourteen members categorized into A and B families with nine EphAs (EphA1–8 and EphA10) and five EphBs (EphB1–4 and EphB6)[4]. The ligands of Ephs, EFNs, are also cell surface molecules with eight members[2]. EFNs can also be divided into A and B family depending on the way they anchor to the cell surface and their affinity to Ephs. There are five EFNAs (EFNsA1–5), and 3 EFNBs (EFNB1-3) [5] .(fig.1.1)

The extracellular domain of Eph is composed of a ligand binding globular domain (LBD), a cysteine-rich region (CRD) and two fibronectin type III (FNIII) repeats, which are followed by a transmembrane (TM) helix[6]. The cytoplasmic domain of Eph is composed of four functional unites with the juxtamembrane (JM) domain that contains two conserved tyrosine (Y) residues, a classical protein tyrosine kinase domain, a sterile α-motif (SAM) and a Psd-95, Dlg and ZO1 domain (PDZ)-binding motif. The PDZ-binding motif is located in the very last 4-5 amino acid residues (XYXV) at the C-terminus followed by a hydrophobic residue in the end [2].

The structures of EFNs vary between A and B families[7]. The ectodomain of both EFNA and EFNB contains a conserved extracellular receptor-binding domain (RBD)[8]. However, EFNBs differ from the membrane-bond EFNAs with transmembrane helix and intracellular domains which contain several conserved tyrosine residues including a PDZ-binding motif. Tyrosine residues of the EFNB intracellular domains can also be phosphorylated upon engagement with Eph receptors. EFNAs, on the other hand, are anchored onto the plasma membrane via glycosylphosphatidylinositol (GPI) linkage [6, 9, 10].

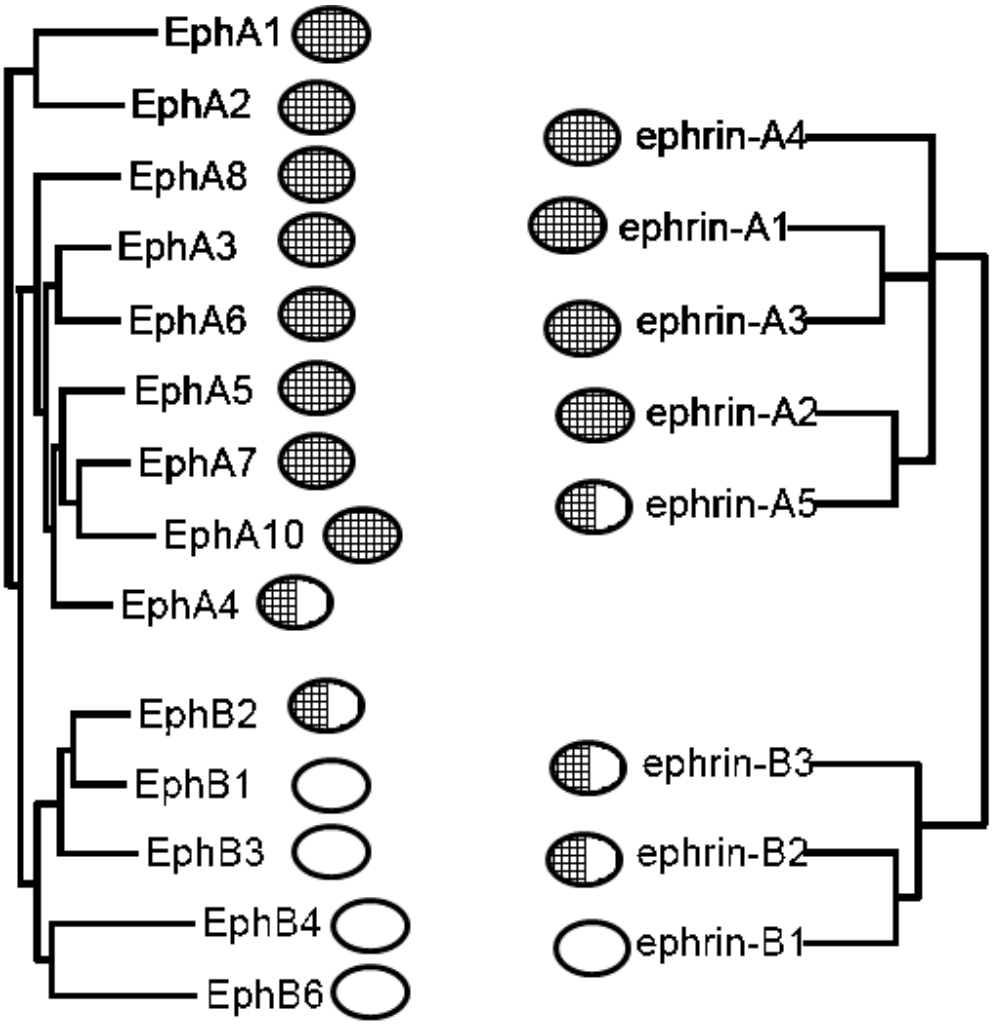

fig1.1 Binding interactions and sequence relationships of Eph receptors and EFNs. Mesh and blank indicate high binding affinity within A and B family, respectively. Half mesh and half blank represent the weak cross reactivity to members of the other family.

I.1.2 Signaling of Eph and EFN

One distinctive feature of Ephs family is that their ligands, EFNs, are also cell surface proteins[2]. Interaction between Eph and EFN relies on direct contact between adjacent cells. Both Eph and EFN are able to transduce downstream signaling to their host cells upon binding to each other. The signal transduced by Eph is called forward signaling, while the signal transduced by EFN is called reverse signaling[6].(fig.1.2)

Eph forward signaling involves EFN-induced clustering, auto-phosphorylation and association with adaptor proteins through intracellular domains [2, 11]. Before EFN binding, Ephs are evenly distributed across the cell membrane and demonstrate minimal kinase activity. Upon binding with the LBD of Ephs, EFNs insert the extended loop into a deep channel of the Eph LBD, while lower affinity interfaces join two Eph-EFN dimers with two adjacent Ephs, assembling into hetero-tetramers and developing into higher-order clusters, which induce auto-phosphorylation of Eph intracellular domain [12]. The extent of EFN aggregation critically influences Eph signaling. EFNs need to cluster to form dimers to oligomers to stimulate tyrosine phosphorylation of Eph intracellular domains and initiate downstream responses [13]. The tyrosine kinase activity of Eph are regulated by the configuration of its JM domain [14]. Two conserved tyrosine residues in the JM domain have been identified as major auto-phosphorylation sites [15]. In a resting state, unphosphorylated JM forms a closed, auto-inhibited conformation. Once being phosphorylated on the JM tyrosine residues, the JM domain releases the kinase domain, allowing it to convert into an active form. Meanwhile, tyrosine phosphorylation creates docking sites for binding domains of adaptor proteins such as Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains [16, 17].

The activated Eph tyrosine kinase domain in turn activates intracellular effector proteins, which modulate cytoskeletal dynamics by regulating small Rho family GTPase activity such as Rac1, Cdc42 and RhoA[18]. The activity of Rho GTPases is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The Rho GEF ephexin1 (Eph-interacting exchange protein 1) binds to Eph constitutively. Prior to EFN binding, Eph-bound dephosphorylated ephexin1 activates RhoA, Rac1 and Cdc42, balancing GTPase activity in the cell [19]. Upon activation of forward signaling, tyrosine phosphorylation of ephexin1 shifts the balance specifically towards RhoA activation.[19] Another Rho GEF Vav2 only binds to phosphorylated tyrosine residue of Ephs when it is activated. Phosphorylated Vav2 promotes local Rac1-dependent endocytosis of the EFN–Eph complex, thereby terminating both forward and reverse signaling [19].

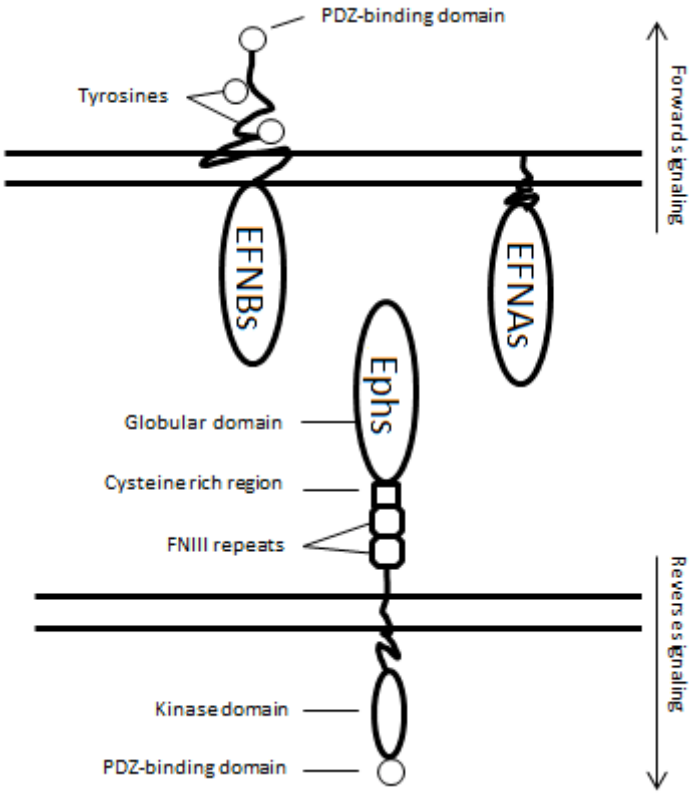

Fig.1.2 Bi-directional signaling of Ephs and EFNs. Ephs and EFNBs are all transmembrane proteins, while EFNA bound to cell membrane via GPI anchor. When cells expressing Eph receptors contact EFN expressing cells, the Eph receptors transduce forward signals into the host cell through their tyrosine kinase activity. Meanwhile, EFNBs transduce reverse signals through auto-phosphorylation and recruitment of adaptor proteins.

RasGAP forms a complex with p-RhoGAP, which negatively regulates the small GTPase Rho. The Ras-Rho complex binds to Eph phosphotyrosine residues by its SH2 domain [15] . In the case of EphB2, activation of EphB2 induces tyrosine phosphorylation of p62dok, a pleckstrin homology (PH) domain-containing protein that recruits RasGAP and the SH2 adapter protein Nck [20]. Nck interacts with serine/threonine kinases of the p2l-activated kinase (PAK) family, and SOS (son of sevenless), an activator of the small GTPases Ras [21]. Upon being recruited to the membrane by Nck, PAK is able to self-active and regulate Jun kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) signaling [22, 23].

Although serving as ligands, transmembrane EFNBs are also able to transduce signal into host cells through their intracellular domains upon binding to Ephs [23]. The intracellular domains of

EFNB1/B2/B3 show high sequence conservation including an almost identical 33 amino acids at the c-terminus which contains three tyrosine residues enabling the recruitment of SH2/SH3 adaptor proteins, a PDZ binding domain, and a D-domain for interaction with Erk/MAPK [24]. The D-domain is located at leucine 293 (L293) in the JM of EFNB3. It has been reported that, Erk2 binds much stronger to D-domain of EFNB3 than Erk1, though both Erk1/2 are able to bind to EFNB3. In neurons, interaction between EFN and Erk has been linked to the regulation of synapse density and the formation of dendritic spines. EFNB3/Erk2 binding retains Erk in the dendrites, thus negatively regulating Erk signaling [25].

Several proteins have been identified that bind via PDZ domain directly such as Glutamate receptor interacting protein (GRIP1), protein tyrosine phosphatase BAS-like (PTP-BL) and PDZ-RGS3 [26-28]. PDZ-RGS3 contains a PDZ domain and a RGS domain [28]. PDZ-RGS3 binds to EFNB c-terminal PDZ binding motif via its PDZ domain constitutively. Meanwhile, its RGS domains act as GAPs for Gα subunits of G proteins to promote the hydrolysis of GTP, thus negatively regulating Gαi, Gαq, and Gα12/13 coupled signaling pathways [29]. However, clustering of EFNBs after activation is pivotal to the regulatory function of PDZ-RGS3 [28]. For example, stromal-cell-derived factor 1 (SDF-1) has been shown to induce cerebellar granule cell migration via CXCR4, a G-proteincoupled chemokine receptor. Only when binding to Ephs could EFNB1 induce inhibition of this chemotaxis via PDZ-RGS3 [28].

PTP-BL contains five PDZ domains and a PTP domain, which interacts with EFNB1 with its fourth PDZ domain [27]. It has been reported that PTP-BL negatively regulates EFNB1 reverse signaling by dephosphorylating tyrosine residues of EFNB1 intracellular domain, which is the target of Src kinase during activation [30, 31] .

Stimulation of EFNB1 leads to the formation of large sphingo-lipid/cholesterol-enriched raft patches [26]. During this process, GRIP1 are recruited into such rafts through binding to the PDZ-binding motif of EFNB1. It has been reported that GRIP1 acts as a multi-PDZ scaffold protein which docks EFN and a serine/threonine kinase. Therefore, the recruitment of signaling molecules by GRIP1 to EFNB complex links EFNB reverse signaling to kinase cascades, propagating signal transduction or remodeling cytoskeletal organization[26].

Upon binding and clustering with Eph receptors, EFNBs also recruit and activate Src family kinases which phosphorylate specific tyrosine residues of the EFNB intracellular domain [32].

Phosphorylated tyrosine residues provide docking site for SH2-containing adaptor proteins, such as Grb4. Through its SH3 domains, Grb4 associates with a set of partners which regulate cytoskeleton dynamics including the Cbl-associated protein (CAP), Ab1, the Abl-interacting protein-1 (Abi-1), and PAK1 [33] .

Dishevelled (Dsh) contains three conserved protein domains: DIX (Dishevelled-Axin), PDZ and DEP [34]. Dsh might binds to the intracellular domain of EFNB1 through interaction with Grb4. Dsh is known to be a downstream molecule of Frizzled (Frz). It has been reported that Xenopus Dsh mediates EFNB1 reverse signaling through the planar cell polarity pathway [35]. Moreover, Dsh has also been implicated in mediating RhoA and Rho kinase activation downstream of EFNB1. Although Dsh binds to EFN-B1 constitutively, such effect only occurs in response to EFNB signaling [34].

Although Eph/EFN system transduces bidirectional signals, Ephs and EFNs may also play independent roles in concert with other cell-surface receptors [3, 36-38]. For example, EFNBs could be phosphorylated on tyrosine in response to activation of growth factor receptor, another family of RTK. Activated fibroblast growth factor receptors (FGFR) inhibit EFNB1 activities by bounding directly to EFNB1 *in cis* and phosphorylating its tyrosine residues [38]. Accumulating evidences have revealed more crosstalk partners of Eph and EFNs, such as Ryk, adhesion molecules as integrins and cadherins, as well as ion channels like NMDA receptor [3]. Recent research from our lab has identified new crosstalk between EFNBs and cytokine receptors IL6R and IL7Rα. Our data demonstrated that EFNB1 and EFNB2 interact with IL7Rα, preventing its internalization upon IL7 treatment, and hence, maintaining IL7R signaling. Similarly, EFNB1/B2 are also critical for transducing IL6R downstream signaling [39, 40].

I.1.3 Physiological role Eph/EFN signaling

I.1.3.1 Role of Eph/EFN in neuron development and injury repair

The Ephs and EFNs were initially identified and mostly studied in central nerve system as axon guidance molecules. Ephs and EFNs are highly expressed in the developing nervous system, where they play important roles in guiding axons and synaptic formation[41].

EphBs selectively promote formation of the spinal synapses and play a critical role in spine maturation, which may involve regulation of cytoskeleton through several GEF for Rho GTPases such as Kalirin, Intersectin, and Tiam1[42]. It has been reported that ectodomain of EphB2 associates with NMDA neurotransmitter receptors promoting clustering at synapses upon EFNB ligation [43]. Moreover, EphB2 also triggers presynaptic differentiation by regulating AMPA neurotransmitter receptor localization through its PDZ binding domain interactions [44]. Knockdown of EphB2 in neuron cultures results in decreased functional synaptic inputs, spines, and presynaptic specializations. On the other hand, expressing EphB2 in non-neuronal cells can drive the formation of presynaptic structures in co-cultured neurons, indicating the influence of activated axonal Eph signaling [44] .

EFNB expression is also identified on synapsis, especially post-synaptically [45]. In cultured neurons, excitatory synapses induced by EFNB3 overexpression are found located on the dendritic shaft. Accordingly, in EFNB3 knockout mice reduced shaft synapses in hippocampal area CA1 have been identified. Moreover, activation of EFNB reverse signaling in cultured hippocampal neurons with EphB2-Fc promotes synapse formation and dendritic spine maturation. Such effect may require recruitment of GIT1 through Grb4 [46].

Ephs and EFNs are also involved in neuron injury reparation[47]. Upregulation of multiple Ephs and EFNs has been detected at sites of nervous system injury. However, their roles during the injury reparation vary. On one hand, EphBs promote the regeneration of injured axons. Interaction of EphB3 expressed in the infiltrated macrophages and EFNB3 on retinal axons promotes axon sprouting in the injured mouse optic nerve. In addition, meningeal fibroblasts that also invade in the injury site express EphB2. Interaction of EphB2 and EFNB2 expressed in astrocytes promotes the segregation of fibroblasts and astrocytes and formation of the glial scar and surrounding basal lamina [48]. On the other hand, EphA4 inhibits nerve regeneration. EphA4 accumulates in both damaged corticospinal axons and astrocytes in injured spinal cord. It has been shown that activating EphA4 forward signaling induces axon retraction and the formation of glial scar in astrocytes, which all inhibit axon regeneration [47, 49].

I.1.3.2 Role of Eph/EFN in bone development

During bone formation, expression of Ephs and EFNs has been identified in chondrocytes, osteoclasts, osteoblasts and osteocytes [2]. EphA4 is expressed in mouse growth plate cartilage as well as in human chondrocytic cell lines [50]. In human articular cartilage cells, expressions of EFNB2 and EphB4 have been reported. In addition, EFNB1 and B2 are expressed on osteoclasts and may stimulate EphB on osteoblasts cells [51].

In humans, *EFNB1* mutations are associated with the craniofrontonasal syndrome (CFNS) which manifests a series of developmental abnormalities such as cleft palate, hypertelorism, frontonasal dysplasia, agenesis of the corpus callosum, and hypoplasia of the maxilla [52, 53] .

In mice, both forward signaling through EphB2/B3 and reverse signaling through EFNB1 are required for skeletal formation [54]. EFNB1 has been reported to be required for correct positioning of the palatal shelves during embryonic development. Deletion of EFNB1 in osteoblast leads to exencephaly due to reduced size of calvarial bones. Lacking of EFNB1 also leads to reduced size and bone mineral density of long bone. Mechanism study further shows that EFNB1 reverse signaling dephosphorylates TAZ within a protein complex, releasing TAZ from the complex to translocate into nucleus and to induce expression of osterix, osteoblastic differentiation and mineralization [55].

During bones remodeling in adults, EphB/EFNB bidirectional signaling coordinates behavior of osteoblasts and osteoclasts [56]. During osteoclastogenesis, cytokines produced by osteoblasts activate the transcription factors c-Fos and NFATc1 in osteoclast precursors, promoting differentiation of osteoclast and also EFNB2 expression. Meanwhile, Ephs expressed by osteoblasts stimulate EFNB2 reverse signaling in osteoclasts, which suppresses osteoclast differentiation through a negative feedback loop that represses *Fos* and therefore *Nfatc1* transcription. The PDZ binding motif has been reported indispensable for such suppression effect, probably through its binding with Dvl2 [56, 57]. On the other hand, osteoblasts also receive stimulations from osteoclasts via EphB4 forward signaling which promotes the differentiation of osteoblasts and new bone formation at sites of resorption by osteoclasts. Forward signaling through EphB4 into osteoblasts enhances osteoblast differentiation via the inhibition of RhoA [57] . Moreover, expression of EFNB2 is also upregulated in osteoblasts upon parathyroid hormone (PTH) or parathyroid hormone-related

protein (PTHrP) signaling, which is required, in coordinate with EphB4, for mineralization by osteoblasts [58]. Therefore, cell-cell contact communication between osteoclasts and osteoblasts mediated by EFNB1/B2 and EphB4 switches bone resorption to bone formation by limiting osteoclast differentiation and enhances osteoblast differentiation.

I.1.3.3 Role of Eph/EFN signaling in angiogenesis

Ephs and EFNs are also expressed in the vasculature, where they play a critical role during angiogenesis, particularly in regulating cell sorting and segregation [59, 60]. Expression of EFNB2 and EPHB4 has been identified in angiogenic endothelial cells (EC) in arterial and venus vasculature, respectively, and mediates arterial-venous vessel segregation and vascular remodeling [59, 61]. The expression pattern of EFNB2 and EphB4 contributes to the control of cell migration with distinct arterial-venous fates. It has been shown that EFNB2 reverse signaling induces endothelial cells migration in response to VEGF or EphB4 and constitute the dorsal aorta [62, 63]. Moreover, EFNB2 phosphorylation occurs exclusively in angiogenic vessels of retina, healing wounds, and tumor [64]. On the other hand, ECs expressing EPHB4 preferentially form the cardinal vein [65].

Although the exact role of Eph and EFN in angiogenesis remains largely unclear, several lines of evidences have implicated their importance. First, EFNB2 deletion in vascular smooth muscle cells leads to spreading defects, focal adhesion lost, and excessive depolarized motility [66]. Second, deletion of EphB4 or EFNB2 are embryo lethal marked with defects in the primary vascular plexus, which suggests their critical role in early vascular development [67, 68]. Consistently, deletion of EFNB2 in the endothelium and endocardium of the developing vasculature and heart demonstrated a similar phenotype [69]. Further study showed that EFNB2 reverse signaling plays a key role in this process [70]. Third, during angiogenesis, recruitment of pericytes is required for proper organization of endothelial cells [66]. EFNB2 has been shown to be essential in maintaining proper vascular architecture through ensuring spatial organization of the pericytes covering microvessels. Deletion of EFNB2 in pericytes *in vivo* leads to extensive hemorrhage in multiple organs including the skin, the lung, the intestine, and kidney, which indicates vascular malformation [66]. Further study using human umbilical vein endothelial cells (HUVECs) *in vitro* established the role of EFNB reverse signaling during cell–cell contacts between pericytes and extracellular matrix in vascular development.

It has been shown that Src-dependent phosphorylation of cytoplasmic domain of EFNBs and downsrtream STAT3-Jak2 signaling is required for extracellular matrix–mediated assembly of endothelial cells and pericytes [64].

In addition to developmental and normal physiological angiogenesis, Eph and EFN are also involved in tumor angiogenesis [71]. In tumor cells and the vasculature of tumors, expression of Eph and EFN can also be detected, which promotes angiogenesis [59, 64, 72] . EFNB2 reverse signaling in tumor endothelial cells, pericytes and smooth muscle cells has been shown to be important for blood vessel assembly, enlargement and decreased permeability both in cell culture and in vivo [63, 73]. It has been shown that EFNB2 signaling promotes the interaction between endothelial cells and vascular smooth muscle cells.[64] Accordingly, EphB4 expressed by tumor cells enhances blood vessel growth through interactions with endothelial EFNB2 [74]. Consistent with these findings, in several mouse tumor models, EFNB2 deletion inhibits tumor growth and angiogenesis [75]. Moreover, similar as normal physiological angiogenesis, intense EFNB2 phosphorylation has been identified at the tumor margins where angiogenesis is most robust [64].

In addition to EFNB2 reverse signaling, EphA2 forward signaling also plays an important role in tumor angiogenesis. EFNA1, the ligand of EphA2, is present in tumor endothelial cells as well as tumor cells. Interaction between EFNA1 and EphA2 leads to activation of PI3k, Vav, and Rac1 leading to regulation of endothelial cell shape and migration [76].

I.1.3.4 Eph and EFN in cancer

Since Eph and EFN function importantly during normal physiological processes in organizing temporally specific cell behaviours, dysregulation of Eph and EFN expression would contribute to cancer progression[77-79]. Eph and EFN expression levels have been correlated with cancer progression, metastatic spread and patient survival. For example, EphA2 is preferentially expressed in malignant breast and prostate cancers and its expression has been linked to increased malignancy and a poor clinical prognosis[76]. In addition, EphB4 is also widely expressed in various cancer cells. Upregulation of EphB4 has been correlated with cancer progression[72]. However, in malignant cancer cell lines and tumor specimens, down-regulation of some Eph and EFN has also been identified. Further studies in colorectal cancer

showed that, after initial up-regulation, expression of Eph is repressed epigenetically or transcriptionally in more advanced stages. However, expression regulation of Eph and EFN is not uniform in cancers. Differential transcriptional regulation has been reported for *EphB2, EphB4* and *EFNB* during colorectal cancer progression. Similarly, inversed expression pattern of EphA2 compared with other EFNA in breast cancer cell lines has also been reported [80, 81].

In colorectal cancer, up-regulation of EphB is attributed to constitutive activation of the Wnt/β-catenin/Tcf pathway [2]. The observed down-regulation of EphB later in advanced colorectal cancers has been linked to hypoxia in the surrounding tissue [81]. Hypoxia induces hypoxia-inducible factor-1 (HIF-1) which competes with Tcf-4 for binding to β-catenin, resulting in suppression of EphB expression. EphB has been reported to be capable of suppressing tumor growth in colorectal cancer. In $Apc^{Min/+}$ mouse model, mutation or losing expression of EphB promotes adenocarcinoma progression due to lack of E-cadherin-dependent spatial restriction by surrounding EFNB expressing epithelial cells [82].

In breast cancer, EphA2 and EphB4 are extensively studied. Both Ephs are widely expressed in human breast cancer cell lines with low level of activation [72, 83]. Overexpression of EphA2 in a human mammary epithelial cell line induces oncogenic transformation without stimulation of EFNs[83]. On the other hand, knockdown of EphA2 or EphB4 has been shown to inhibit the tumorgenicity of several cancer cell lines. Several lines of evidences have suggested that low versus high level of activation of Ephs leads to converse outcome[11]. In the absence of forward signaling, aberrantly expressed Eph may crosstalk with oncogenic signaling pathways, such as EGF receptor family members, to enhance tumor cell proliferation and motility[37]. One the other hand, activation of EphA2 or EphB4 readily suppresses the survival and tumor formation of human breast cancer cells in xenograft model possibly due to inhibition of downstream Ras-Erk, PI3K-Akt and Abl-Crk pathways [83, 84].

In melanoma, the role of Ephs is contradictory [85, 86]. EphA forward signaling has been shown to promote tumor cell proliferation and be associated with formation of blood vessel-like structures [87]. In contrast, though EphA2 is upregulated in both mouse and human skin carcinomas, deletion of EphA2 in tumor cells leads to their elevated growth and invasion[86]. Similar to EphB/EFNB interaction in breast cancers, interaction between EphA2-expressing tumor cells and EFNA1-expressing surrounding tissue restricts expansion of the EphA2-

positive tumor cells by inhibiting Ras-dependent pathways [86]. On the other hand, activation of EphB4 by membrane-bond EFNB2 or soluble EFNB2-Fc yields different outcomes. While co-expressed EFNB2 promotes amoeboid via RhoA activation in EphB4-expressing cells, EFNB2-Fc inhibits proliferation, survival, migration, and invasion of human MDA-MB-435 cell line both *in vitro* and in a mouse xenograft model. Such effect may possibly involve the Abl and Crk pathways[72] .Moreover, EFNB2 reverse signaling has also been associated with β1-integrin signaling and promotes cell adhesion and migration [78].

I.2 Roles of Eph/EFN in immune system

A few Ephs and EFNs are also expressed in lymphoid organs and lymphocytes, which implicates their possible roles in immune system [88]. Given the cell surface expression of Ephs/ EFNs, it is reasonable to predict that they may function in immunobiology where cell-cell direct contact is critical, such as thymocyte development in thymus, and T migration and differentiation in lymphoid organs, where T cells encounter antigen-presenting cells (APC).

It has been reported that interfering Eph-EFN interactions with EphB2-Fc or EFNB1-Fc leads to hampered thymocyte development and elevated apoptosis in thymic organ culture [89]. Moreover, multiple researches on mice deficient in several EphA and EphB family members all reported dramatic decrease of thymocytes and peripheral T cells due to disorganized thymic architecture [90] . Previous work in our lab also demonstrated that, deletion of EFNB1/B2 in T cell compartment leads to not only diminished T cell populations but also compromised T cell functions[40] . All the evidences suggest that Eph/EFN interaction is critical for thymic structural organization and T cells development.

In addition to their roles in T cell development, Eph/EFN have also been shown to modulate T cell receptor (TCR)-mediated responses[88] . Several reports have shown that solid phase EFNB1/B2/B3 are capable of promoting TCR response via activation of EphBs. Activated EphB forms a clustering cap on T cells together with TCR in aggregated lipid rafts, and, therefore, lowers the activation threshold of T cells upon TCR ligation. Moreover, EphB ligation also promotes T cell proliferation, cytokine production, and cytotoxic T cell activity [91-93] .

EphB6 is one of the most extensively investigated members of EphB in the immune system [94-96]. It is highly expressed in human mature T cells. In mouse lymphocytes, EphB6 is mainly expressed in double positive (DP), a fraction of mature CD4+ T cells, and CD8+ T cells. Interestingly, expression of EphB6 is under a dynamic balance through rapid synthesis and shedding [96, 97] . The intracellular domain of EphB6 lacks kinase activity. However, stimulation of T cells with anti-EphB6 antibodies or EFNBs still leads to increased tyrosine phosphorylation and downstream signaling, which may be due to association of EphB6 with EphB1 and EphB4 co-expressed on the same cell [88-90] . It has been suggested that EphB6 is critical for enhancing TCR signaling. Our lab have previously reported that, T cells from EphB6-/- mice show impaired TCR signaling, proliferation, and cytokine secretion in vitro. Moreover, EphB6-/- mice show compromised immune responses although their T cell numbers are normal. Further study showed that, EphB6 ligation enhances suboptimal TCR signal and lead to drastic T cell proliferation, accompanied by enhanced production of several cytokines, such as interferon (IFN)-γ, interleukin (IL)-6, IL-10, TGF-β, tumour-necrosis factor (TNF)-α, and GM-CSF, but not IL-2 and IL-4 [95] . Such effect involves up-regulation of the p38 and p42/44 MAP kinases [91-93] .

On the other hand, excessive EphB signaling by EFNB1 and EFNB2 co-stimulation demonstrates inhibitory effect on TCR-mediated responses in T cells, which is most likely mediated by EphB4. It has been shown that EphB4 forward signaling inhibits T cell proliferation by targeting Lck [98, 99] . High concentration of EFNBs induces EphB4 phosphorylation which, in turn, recruits SHP1. SHP1 dephosphorylates protein tyrosine kinase Lck at Tyr-394, and hence, negatively regulates T-cell signaling [100, 101] .

Moreover, research in our lab has reported that, EFNBs can cluster with several cytokine receptors and this is critical for downstream signaling. We have shown that EFNB1 and EFNB2 co-cap with IL-6R and IL-7R ensuring their signaling upon cytokine engagement [39, 40] .

In addition to EphBs and EFNBs, EphAs and EFNAs expression are also detectable in thymocytes and T cells [88, 102] . Similar to their cousins, EphAs and EFNAs are involved in TCR signaling modulation. EFNA1-Fc inhibits IL-2 secretion and induces apoptosis in DP cells under strong TCR stimulation, which suggests their role in negative selection. In CD4+

cells, ligation of EFNA1 with antibodies has been reported to suppress TCR-mediated responses, suggesting that EFNA1 reverse signaling may play a role in this process. Furthermore, EphA/EFNA signaling regulates migration of thymocytes in the thymus and T cell trafficking in responses to chemokine stimulations, such as SDF1-α and integrin-dependent adhesion. Mechanistic study has revealed that upon EFNA1 ligation, phosphorylation of Lck in association with proline-rich tyrosine kinase 2 (Pyk2) and Vav1 is critically required. Moreover, involvement of the PI3K pathway and Rho GTPase downstream signaling are also implicated after EFNA1 ligation [88, 103, 104] .

I.3 T cell development in the thymus

T cells and other lymphocytes arise from the same origin, the hematopoietic stem cells (HSC) in the bone marrow. The committed precursors enter circulation and arrive in the thymus initiating the T cell development program [105, 106] . The thymus is the major organ for T cell development and maturation which guarantees the production of proper reactive T cells [107-109] .

I.3.1 T cell development and lineage selection

Development progress of T cells can be divided into several stages according the expression pattern of CD4 and CD8 co-receptors, which can be defined as CD4-CD8- double negative (DN), CD4+CD8+ double positive (DP) and CD4+/CD8+ single positive (SP). The DN stage can be further segmented by expression of CD25 and CD44: DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; and DN4, CD44-CD25- [110] . The whole procedure consists several important events, including the rearrangement and expression of TCR genes, population expansion, positive and negative selection, and acquisition of functional capabilities [111, 112] . Both αβ and γδ T cells are derived from the common precursors referred as early thymic progenitors (ETP). The ETP migrate to thymus cortex from bone marrow, and gradually commits to the T cells linage during DN stage [113] .

Lineage commitment is the first essential event during T cell development. It has been established that the lineage commitment requires the sustained repression of the expression of genes characteristic of the alternative lineage. In early stages of ETP until DN2, signalling of

Notch 1, upon interaction with its ligand Delta-like 4 (DL4) expressed on the thymic stroma, is required to guarantee the T lineage commitment [114-116] . Sustained Notch1 signalling promotes T lineage specific gene expression and cell survival. However, Notch1 alone is not sufficient for T lineage commitment. Other transcription factors, including Runx1, GATA-3 and E-box proteins, are required to cooperate with Notch1 to initiate T cell differentiation [117] .

Further development of T lineage committed DN thymocytes requires expression of RAG-1 and RAG-2 recombinase to initiate the rearrangements of three TCR gene loci–*Tcrb*, *Tcrg* and *Tcrd*. Meanwhile, TCR-α is encoded by a non-rearranging locus. Among them, *Tcrb* starts rearrangement first, which involve the deletion of intron and joining of segments of $V_\beta D_\beta J_\beta$ genes as well as C_β gene. The further transcription and translation procedures yield a rearranged TCR_β [118] . The successfully rearranged TCR_β pairs with invariant pre-Tα chain (pre-TCR), resulting in enhanced proliferation, followed by entry of the double positive (DP) stage. With the rearrangement of TCR_β, *Tcrg and Tcrd* gene expression are excluded. T cells bearing rearranged *TCRb* gene next go through the verification checkpoint known as β-selection, before entering DP stage. Survival in the β-selection requires the signalling through a pre-TCR complex with a properly rearranged TCRβ chain, CD3γ, δ, ε, ζ chains and the pre-Tα. The pre-TCR complex is capable of transmitting signals through its intracellular intermediates without the requirement for extracellular ligands [117] .

On the other hand, successful rearrange the *Tcrg* and *Tcrd* loci leads to expression of the γδ–TCR and exclusion of *Tcra* and *Tcrb*. Similar to TCR αβ thymocytes, thymocytes adopted γδ direction also go through the check point for TCR rearrangement verification at DN3. However, unlike the αβ cells, there is no pre-TCRγδ. The DN3 checkpoint verifies signalling by mature TCRγδ complexes. Moreover, γδ lineage differentiation is independent on Notch signalling [119] .

It has been suggested that distinct strength of signals transmitted by pre-TCR or TCRγδ complexes could guide the αβ /γδ decision at DN3 stage. Strong signals transmitted by γδ TCR promote γδ lineage choice, whereas the weaker signal from pre-TCR guides the cells to αβ direction [120] . This notion is supported by the evidence that introducing signalling-defective CD3ζ chain into DN3 cells which disrupt TCRγδ signalling leaded to the generation

of TCRγδ- DP thymocytes [121] . Another study using a transgenic TCRγδ of defined antigen specificity have shown that thymocytes expressing a transgenic TCRγδ receptor are directed to γδ lineage in mice expressing the corresponding ligand, but they adopt an αβ fate when the ligand is absent, suggesting that the interaction between TCRγδ and its ligand is necessary to ensure the signal strength and, hence, direct the cells to γδ lineage [122] .

In addition to TCR signalling, environmental signals have also been proposed to play a role in directing γδ/αβ lineage decision before the DN3 checkpoint by affecting TCR gene rearrangement. The strong IL-7 signal is required for *Tcrg* but not for *Tcrb* gene rearrangement. DN2 cells can be subdivided based on the expression level of IL-7Rα, and IL-7Rα^{hi} cells gave rise to higher proportion of γδ T cells than IL-7R$^{low/-}$cells [123] . Whether this phenomenon is caused by pre-commitment or high frequency of *Tcrd* rearrangements in IL-7Rα^{hi} cells remains to be elucidated.

At DN3, several transcriptional targets of TCR or pre-TCR signals play crucial role in facilitating cells to overcome this check point [124] . Further development of DN3 cells are halted by E-box binding proteins E2A and HEB [125] . It has been proposed that cells overcome this block by two complementary mechanisms. One is to reduce the expression E2A or HEB coding gene. The other is to increased expression of Id-family molecules, which inhibit the expression of E-protein [126] . Id3 is a member if the Id family, which is a transcriptional target of both pre-TCR and TCRγδ signals. It has been proposed that different signal strength mediated by pre-TCR or TCRγδ decides the extent of Id3 up regulation, which distinguishes the lineage decision [127] . High Id3 expression alone in γδ cells is sufficient to down regulate E-box binding protein level, hence, facilitating overcoming the E-protein blockage. However, cells expressing Id3 at lower level in response to pre-TCR signalling require the cooperation of Notch1 signalling in down regulating E-protein expression and compete with E-protein on *Tcra* regulation region [122, 127-129] .

Another important effector is the Egr transcription factor family members (Egr1 and Egr3). Egrs are triggered by both pre-TCR and TCRγδ signals. Over expression of Egr1 interfered with αβ lineage development both in culture and in vivo[122]. It has been suggested that high expression of Ergs promotes γδ T cell development, possibly by facilitation of Id3 up regulation [127] .

In addition, it has also been proposed that, HMG transcription factor Sox13 is important for the development of some γδ T cells by inhibiting the generation of αβ T cells, possibly by antagonizing the function of TCF1. TCF1 functions to favour αβ gene rearrangement while inhibiting γδ gene rearrangement [130] . Therefore, by antagonizing TCF1, Sox13 contributes to γδ lineage decision.

Thymocytes, which successfully passed the β-selection, initiate CD4 and CD8 expression, becoming CD4+CD8+ DP thymocytes. Meanwhile, *Tcra* gene is rearranged resulting in the final expression of TCRαβ complexes on cell surface. Three effectors contribute to the generation of DP cells from β-selected cells. First, Runx1 is necessary for the proliferative burst that follows β-selection [131] . Second, RORγt, encoded by the Rorc gene, promotes cell survival by up regulating expression of the anti-apoptotic protein Bcl-xL [132] . Lastly, the high mobility group (HMG) protein, TCF1, cooperates with its partner β-catenin to suppress γδ rearrangement and prompt generation of DP cells [120, 133] . The next and final stage of αβ DP cells maturation in thymus is positive and negative selection. DP cells that express TCRαβ which recognizes peptide-self-MHC complexes on the cortex thymus epithelium are prompt to survive from apoptosis. The selected cells are able to recognize foreign antigens loaded on the same self-MHC of APCs in periphery. These cells continue to go through negative selection. This step ensures the elimination of self-reactive cells, as well as generation of CD4+ or CD8+ cells matching their MHC specificity. Thymocytes restricted to MHC class II adopt CD4 fate while MHC class I–restricted thymocytes become cytotoxic CD8 cells. This procedure leads to clonal deletion of thymocytes with receptors of the highest avidity for self-antigens, which provides central tolerance [134, 135].

I.3.2 Thymic epithelial cells guide the development of thymocytes

The thymus provides a microenvironment for thymocyte development. During the whole developmental process, thymocytes are at close proximity of thymic stroma and migrate within the thymus since the arrival of lymphoid progenitor cells in the thymus. The structure of the thymus is highly compartmentalized and composed mainly of two kinds of epithelial cells which form cortex and medullar scalfolds separately [136] . (Fig1.3)

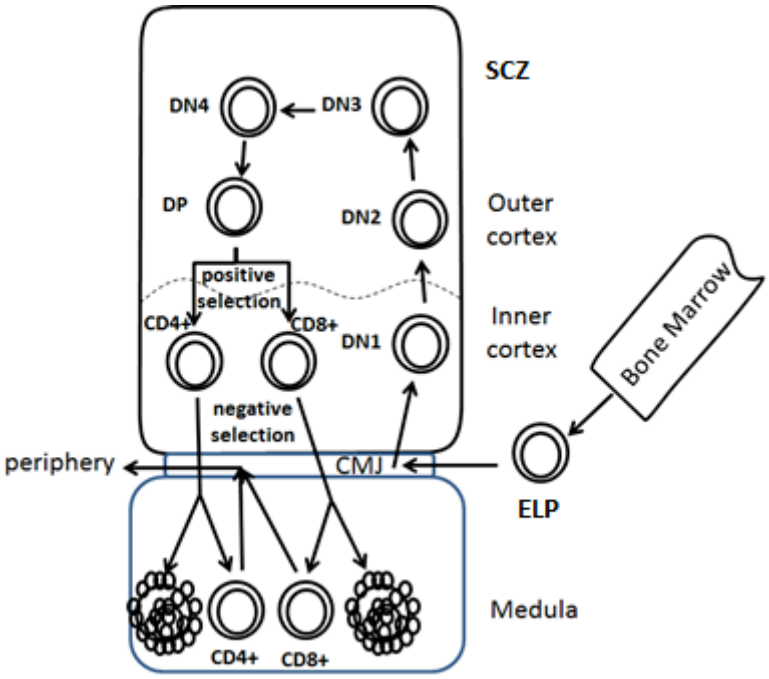

Fig 1.3 T cell migration during development in thymus. Bone marrow-derived early lymphoid progenitors (ELP) enter the thymus through circulation at the corticomedullary junction (CMJ) and migrate to inner cortex while differentiating into DN1 and DN2 cells.
DN2 cells further differentiate into DN3 cells in the mid and outer cortex. DN3 cells accumulate in the subcapsular zone (SCZ) undergoing proliferation and differentiate to DN4 cells. The developing thymocytes migrate backwards to inner cortex layer and medullar while further developing into DP stage. DP cells undergo positive selection and differentiation into SP cells during migration through the cortex. The surviving SP cells enter the medulla, where they undergo negative selection and final functional maturation. Finally, mature T cells leave the thymus and enter theperiphery.

Early progenitors arrive at the thymus at cortico–medullary junction (CMJ) from circulation. Seeding of the progenitors requires interaction of P-selectin expressed by thymic endothelium and its ligand PSGL-1 expressed on the progenitors. Mice lack PSGL-1 demonstrated decreased intrathymic progenitors and increased empty niches for prothymocytes [137] . After successful seeding into thymus, it is critical for the progenitors to efficiently relocate from the CMJ to the outer cortex to initiate thymocyte development. Several chemokines secreted by

the cortex and medullar epithelial cells have been reported to guide this movement, which involves CXCL12, CCL19, and CCL21 [138-140] .

CXCL12 has been reported to be critical in guiding the migration of developing thymocytes in the cortex [141-143] . Expression of CXCL12 receptor, CXCR4, has been identified in all the DN subpopulations as well as a part of the DP cells, which implicates the requirement of CXCR4 in mediating the migration of thymocytes at the DN and DP stage. It has been demonstrated that CXCR4-deficient thymocytes fail to migrate from CMJ to deep cortex and unable to differentiate past the DN1 stage. Notably, expression of CXCL12 is largely homogenous in the cortex, indicating that CXCL12 signal alone may not be enough to polarize thymocyte migration all the way across the cortex to the capsule [138] . On the other hand, later studies have revealed that CXCR4 functions also as a co-stimulator in facilitating thymocytes differentiation during β-selection [144] . In an *in vitro* co-culture system, which uncouples the migration function of CXCR4 signalling, it has been shown that a loss of CXCR4 signalling results in partial blockage of thymocyte development at β-selection [145] .

CCL19 and CCL21 are predominantly expressed in the thymic medulla. However, expression of their receptor, CCR7, has been identified in both DN2 and DP stages [139] . In DN2 thymocytes, CCR7 has been shown to function with CXCR4 in guiding the exit of CMJ. A loss of CCR7 signalling leads to partial blockage of DN1-2 cells to exit CMJ [140]. On the other hand, CCR7 signalling is also critical in mediating a reversed directional chemotaxis of the positively selected thymocytes from cortex to the medulla [146] .

Thymic stromal cells do no only secrete chemokines to guide thymocytes during their development. They also express proteins on their cell surface for the purpose of positive and negative selection as well. Cortex thymic epithelial cells (cTEC) express peptide-MHC complexes, which could bind to the TCR of developing thymocytes. DP thymocytes that bind to peptide-MHC at low-avidity are induced to receive survival signals and continue to differentiate into SP thymocytes [147]. In addition to positive selection, the peptide–MHC expressing cTECs contribute also to the generation of nTreg cells. Typically, expression of MHC classII by cTEC is critical for the nTreg generation in the thymus[148] .

Thymocytes survived from positive selection migrate into the medulla to undergo negative selection and further functional maturation under the guidance of mTECs [149] . mTECs

express a panel of tissue-restricted self-antigens randomly, which mimic the peripheral tissues, to ensure that no self-reactive thymocytes will be released into the circulation [150] . Expression of tissue-restricted self-antigens has been reported to be largely regulated by autoimmune regulator (AIRE), a deficiency of which causes autoimmune polyendocrinopathy-candidiasis-ectodermaldystrophy (APECED) in both human and mice due to a failure of central tolerance [151-153] .

Although thymic stromal cells provide support and guidance to developing thymocytes, the crosstalk between thymocytes and stromal cells also regulates stromal cell organization. It has been reported that the differentiation stage of thymocytes contributes to the regulation of cortical environment in the thymus. Mice deficient of thymocyte development at an early stage demonstrate TECs arrested at an immature stage, which express both keratin 5 and keratin 8[154] . However, normal cortex organization is presented in the thymus of mice with deficiency of thymocyte development beyond the DN3 stage [154] . In addition, overexpression of CD40L in thymocytes alters the balance of cortex and medulla [155]. Meanwhile, knockout of CD40L or CD40 greatly reduces medullar area in the thymus [156] . Similarly, RANKL expressed by Vγ5 γδ T cells and post-selection αβ T cells in the thymus contributes to maturation of mTEC [157] . All the above evidences demonstrate that in the thymus the development of thymocytes and thymus epithelial cells are inter-dependant.

I.3.3 The T helper cells

T lymphocytes play important roles in host defence against foreign pathogens. $CD4^+$ effector T cells differentiate into various subtypes depending on cytokine stimulations and coordinate different immune responses upon antigen engagements during activation [158] . The concept of different types of effector T lymphocytes arises from the observation of inversed relation between delayed type of hypersensitivity (DTH) and antibody levels upon different doses and forms of antigens [159] . Later research using propagating cloned lines of T cells and assays for cytokines leads to the identification of two subtypes of $CD4^+$ effector T cells with distinct profile of cytokine secretion, namely Th1 and Th2 [160] .

I.3.3.1 Th1 in the immune system

Th1 is not a pre-committed lineage of $CD4^+$ cells in nature. Rather, Th1 is derived from mature naive $CD4^+$ T cells with stimulation of IL-12 via the activation of STAT4 [161] . IL-12 is secreted by activated macrophages and dendritic cells in response to intracellular pathogens [162] . Later research has identified a T-box protein expressed in T cells (T-bet) as the hallmark transcription factor that mediates INF-γ expression. Retrovirus induced expression of T-bet in a number of cell lines and primary T cells leads to the expression of INF-γ. Moreover, T-bet functions not only in controlling INF-γ expression, but also in switching the differentiation program of naive T cells as well. Overexpression of T-bet in naive T cells under a Th2 condition readily induces INF-γ expression and represses the expression of Il-4, Il-5 and Il-13, which are the hallmark cytokines of Th2 [163] . It has been reported that T-bet is required to sustain the Th1 lineage throughout the Th1 life cycle by two different mechanisms. During the initiation stage of naive $CD4^+$ T cell differentiation, TCR signalling promotes the interaction between T-bet and GATA-3, the hallmark transcription factor defining the Th2 lineage, via Itk. Binding of T-bet with GATA3 prevents it from activating Th2 signature genes such as *Il4* and *Il5* [164] . After Th1 lineage is fully committed, T-bet is involved in suppressing the *Socs1*, *Socs3* and *Tcf7* genes directly by recruiting the transcriptional repressor Bcl-6 to their promoters [165] .

Thl participates in the elimination of intracellular microbes by producing IL-2, IFN- γ, and tumour-necrosis factor TNF- α [166] . IFN-γ is the principle effector cytokine of Th1, which mediates phagocyte-mediated defence against infections by promoting both phagocytosis and microbiocidal ability of macrophages. Moreover, IFN-γ stimulates the production of IgG which is involved in the opsonization and phagocytosis of particulate microbes. In addition, together with IL-2, IFN-γ also promotes $CD8^+$ T cells differentiation into active cytotoxic cells. On the other hand, if Th1 is inappropriately activated, their proinflammatory cytokines released often cause inflammation and tissue injury [162]. Th1 and related cytokines have been proven to be involved in the pathogenesis of granulomatous inflammation, arthritis and colitis [167] . For example, increased level of IL-12 has been associated with human Crohn`s disease as well as animal colitis models [168] . Moreover, neutralizing IL-12 largely suppresses the intestinal inflammation. [169] Further, T cells with deficient STAT4 or T-bet failed to induce

colitis in an adoptive transfer model [170, 171] . All the evidences clearly indicated the role of Th1 in this disease.

To minimize the aberrant side-effects, Th1 responses are under tight regulation, typically by IL-10. IL-10 was originally identified as a Th2-specific cytokine, which inhibits Th1-mediated responses [172] . Later researches have broaden the panel of IL-10-producing cell to Treg cells, macrophages, dendritic cells (DC), B cells, eosinophils, and mast cells [173] . IL-10 exerts its suppressive function by inhibiting the expression of IL-12, MHC-II, and co-stimulatory molecules by macrophages and DCs [174, 175] . Mice lacking *Il10* autonomously develop colitis due to failed regulation of unwanted Th1 responses against the gut flora [176] . Conversely, the *Il10*$^{-/-}$ mice demonstrate increased resistance to a panel of intracellular pathogens as well as accelerated virus clearance [177] . Interestingly, Th1 itself has also been reported to produce IL-10, which suggests an auto-regulation mechanism of Th1 in preventing tissue injury during inflammatory responses [178] . However, such mechanism was reported to be utilized by some pathogens to cope with Th1 attacks. As a matter of fact, elevated levels of IL-10-producing $CD4^+$ and $CD8^+$ T cells have been observed in the patients with persistent virus infections such as hepatitis C virus (HCV), human immunodeficiency virus (HIV), and Epstein-Barr virus [179-181] . The switch from a proinflammatory mode of Th1 to an inhibitory mode is believed to be due to anergy of the Th1 during a chronic antigenic challenge [182] .

I.3.3.2 Th2 in the immune system

Th2 is the other T helper cell lineage identified along with Th1, featuring the secretion of IL-4, IL-5, IL-13 as well as IL-10 in response to antigen stimulation [183] . Similar to Th1, Th2 is also differentiated from naive $CD4^+$ T cells.

Th2 differentiation is initiated by IL-4R signalling. Binding of IL-4 to IL-4R on activated $CD4^+$ T cells leads to dimerization of IL-4R subunits followed by phosphorylation of their cytoplasmic tails by the Janus Kinase (JAK) family of tyrosine kinases. Subsequently, the phosphorylated tails recruits and phosphorylates STAT6, which then translocates into nucleus and activates transcription of GATA-3 and in tandem, a variety of Th2 hall mark cytokines including IL-4, IL-5, as well as IL-13 [184] . STAT6 does not only initiate Th2 lineage polarization, but also contributes to the maintenance of Th2 lineage by repressing Th1-associated genes. It has been reported that STAT6 directly binds to some loci of Th1-featured

genes, for example the Il18r1–Il18rap locus, and promotes a more condensed chromatin configuration, which epigenetically represses the Th1 potential [185] .

One of the major functions of GATA-3 is to initiate IL-4 expression epigenetically. *Il4* locus is constitutively repressed by methyl-CpG-binding domain protein 2 (MBD2) and nucleosome remodelling and histone deacetylase (NURD) in naive or other committed $CD4^+$ T cells. Upregulation of GATA-3 mediates disassociation of MBD2 from the *il4* locus, leading to chromatin changes, which initiates IL-4 transcription [186] . Moreover, GATA-3 binding sites have been identified in the *Il5* and *Il13* loci, which suggest a direct transcriptional function of GATA-3 on these cytokines [187, 188]. It has been reported that *in vitro* deletion of GATA-3 abolishes Th2 cell differentiation, while Th1 differentiates autonomously even in the absence of IL-12 and INF-γ. Interestingly, deletion of *Gata3* from committed Th2 cells fails to diminish IL-4 production but totally abolishes the expression of IL-5 and IL-13, which indicates that GATA-3 may serve largely to alter the chromatin structure at the *Il4* locus [189]. Another line of evidence has shown that the transcription factor c-Maf, independent from GATA-3, is selectively critical for IL-4 but not IL-5 or IL-13 production [190].

IL-2 is another critical cytokine required for successful Th2 differentiation. IL-2 activates STAT5 and subsequently induces IL-4 expression even in the absence of IL-4Rα and STAT6. This implicates IL-2 in the process of Th2 priming [191]. Neutralizing IL-2 in Th2 cultures greatly reduced IL-4 production, without affecting GATA-3. It has been reported that IL-2-STAT5 pathway functions independently of IL-4-STAT6 singling, as overexpression of GATA-3 failed to induce IL-4 expression in $STAT5^{-/-}$ cells [192] .Therefore, both GATA-3 expression and STAT5 activation are required for Th2 differentiation.

It is noteworthy that, development of Th2 largely depends on IL-4 signalling. However, Th2 itself is the major source of IL-4. It has been suggested that in vivo Th2 responses is initiated by a low level of endogenous GATA3, which allows the initial secretion of a small amount of IL-4 for the initial activation Th2. IL-4 is required to accumulate at the site of infection to reach a necessary threshold for a full induction of Th2. Thereafter, Th2-mediated responses become increasingly pronounced with repeated T cell stimulation [191, 193].

Th2 cells have been shown to play important roles in mucosal immunity and in host defence against extracellular parasites, such as helminthes and nematodes [194]. Th2 cells stimulate the B cells to produce high levels of IgM and IgG [195]. Moreover, IL-4 promotes B-cell to switch

from IgG to IgE production and subsequently initiate IgE-dependent, mast-cell-mediated allergic reactions [196]. In addition to IL-4, IL-5 activates eosinophil in responses to helminths [197].

On the other hand, some Th2 cytokines demonstrate immunoregulatory activities. For example, IL-13 inhibits the activation of macrophage by IFN-γ [198]. Meanwhile, as is discussed in the Th1 section, IL-10 suppresses Th1-mediated inflammation [172]. Considering the kinetics of Th2 responses, Th2 cytokines appear late in immune responses, and serve to limit the injurious consequences of Th1-mediated protective immunity.

Though Th2 possesses immune regulatory functions, aberrant Th2 response will lead to allergic disorders, typically atopic asthma [194]. Experiments on patients of atopic asthma show significant activation of IL-3, IL-4, IL-5 but not INF-γ in the bronchoalveolar lavage (BAL) after allergen challenge [199]. Later, higher level of STAT6 and GATA-3 expressing cells are also observed in asthmatic patients' bronchi [200]. Consistent with human evidences, increased amounts of Th2 type cytokines, i.e., IL-4 and IL-13, are identified from the lung of mice with deficiency in T-bet which is the signature transcription factor of Th1 and the antagonist of Th2 responses. Moreover, in mouse asthmatic models, IL-4 or STAT-6 deficiency protects the mice from Th2-mediated bronchial inflammation and airway hyper-reactivity (AHR) [201, 202]. Similarly, this protection is also observed in mice with transgenic dominant-negative form of GATA-3 in T cells [202]. All the evidences suggest the involvement of Th2 in the asthmatic pathogenesis.

I.3.3.3 Th17 in immune responses

Th17 T cells are another helper T cell lineage that regulates inflammation via production of the hallmark cytokine IL-17A and IL-17F [203]. Although IL-17A is commonly called IL-17, the IL-17 family consists of six structurally related cytokines, namely, IL-17A to IL-17F [204]. (Table 1.1) IL-17A and IL-17F share the highest similarity in sequence while the sequences of IL-17B/C/E show high diversity from IL-17A/F [205, 206]. Unlike IL-17A/F, which are secreted mainly by Th17 cells, IL-17B/C/D/E are secreted by a variety of other cells in tissues [207-210].

Th17 plays important roles in host defense against pathogenic infections at inflammatory sites by secreting various cytokines such as IL-17, IL22, and IL-23. One of the direct functions of IL-17A is to induce differentiation of $CD34^+$ progenitor into neutrophil progenitors by

stimulating GM-SCF secretion of bone marrow stromal cells [211]. Correspondingly, IL-17RA deficient mice showed susceptibility to *K. pneumonia* pulmonary infection with defective GM-CSF production, delayed neutrophil recruitment in the lung and high mortality after infection[212]. IL-2 amplifies IL-17 responses by supporting Th17 maturation. Other studies demonstrated that IL-17 deficiency or IL-23 deficiency also renders mice susceptible to infection of the gram-negative bacteria *Bacteroides fragilis,* parasite *Toxoplasmosis gondii*, fungi *Candida albicans*, as well as *Mycoplasma*[213-216].

Table 1.1 The IL-17 family

Family member	Functions	Expressing cells
IL-17A	Recruitment of neutrophil, autoimmune pathology,	Th17 cells, CD8 T cells, γδ T cells, NK cells, NKT cells
IL-17B	Inducing TNF and IL-1β expression	Cells in gastrointestinal tract, pancreas and neurons
IL-17C	Inducing TNF and IL-1β expression in monocytes	CD4 T cell, DC, Macrophage in the inflammatory sites
IL-17D	Inducing expression of IL-6, IL-8, and GM-CSF in endothelial cells	Cells in the muscles, brain, heart, lung, pancreas and adipose tissue
IL-17E	Enhancing Th2 immune responses, inducing IgE production, contributing to the host defense against nematodes and allergic disorders	Th2 cells, mast cells, intraepithelial lymphocytes, eosinophils, basophils, and NKT cells
IL-17F	Recruitment of neutrophil, immunity to extracellular pathogens	Th 17 cells, CD8 T cells, γδ T cells, NK cells, and NKT cells

Like other T helper cells, Th17 can be induced from naïve $CD4^+$ T cells. IL-23 is the first cytokine which has been shown to be capable of inducing IL-17 secretion on activated $CD4^+$ T cells and critical for Th17 generation *in vivo* [203, 217]. However, since naïve $CD4^+$ T cells lack

IL-23R, it has been suggested that there exists another pathway in guiding the naïve $CD4^+$ T cell to polarize into Th17. In fact, although incapable of initiating Th17 differentiation from Naïve $CD4^+$ cells, IL-23 is required for fully maturation of Th17 cells and their pro-inflammatory functions. Later research demonstrated that pro-inflammatory cytokine IL-6 along with TGF-β are required to induce IL-17 expression in naive $CD4^+$ T cells [218, 219]. During Th17 differentiation, the retinoid orphan nuclear receptor (RORγt) is the key transcriptional marker in both mouse and human which critically depends on IL-6-induced activation of Signal transducer and activator of transcription3 (STAT-3) [220, 221]. RORγt expression in naïve $CD4^+$ T cells is essential for the cells to adopt a Th17 phenotype which express IL-17A, IL-17F and IL-23R. Deficiency in RORγt renders reduced endogenous Th17 cells and protects mice from a multiple sclerosis model, experimental autoimmune encephalomyelitis (EAE). In contrast, over-expression of RORγt promotes Th17 development [222]. TGF-β is also critically required in Th17 induction. The exact cellular source of TGF-β essential for the Th17 differentiation is still largely uncertain. DCs upon stimulation with zymosan and *M. tuberculosis* are capable of secreting sufficient TGF-β to induce Th17 *in vitro* [223]. However, another line of evidence demonstrated the importance of the T cell source of TGF-β in mice with $CD4^+$ T cell-specific deletion of TGF-β. The KO mice failed to yield Th17 cells even after immunization with adjuvant containing Mycobacterium [224]. Treg cells are another source of TGF-β, but the role of Treg in induction of Th17 remains to be elucidated. Interestingly, differentiating Treg cells require TGF-β in common with Th17. When Naïve $CD4^+$T cells are exposed to TGF-β, RORγt and FOXP3 are co-expressed by the cells. Depending on the presence of IL-6, FoxP3 gene could repress the expression of RORγt or be repressed, which yields Treg cells or Th17 cells [225].

In addition to its anti-infection functions, Th17 also plays important roles in autoimmune pathogenesis. In rheumatoid arthritis (RA), increased levels of IL-17, IL-22 as well as IL-23 have been identified in the sera and synovial fluids of RA patients [226, 227]. In a mouse model for autoimmune arthritis, collagen-induced arthritis (CIA), IL-17 is directly involved in cartilage and bone erosion. Blocking of IL-17A at early stage or using IL-17A-deficient mice largely attenuates the disease onset [228, 229]. Similar findings have also been reported in the cases of other autoimmune diseases, such as in central nerve system (CNS) of multiple

sclerosis (MS) patients [230], in skin lesion of psoriasis patients [231], as well as in gut mucosa of inflammatory bowel diseases (IBD) patients [232]. Therefore, targeting the IL-17 pathway is a promising therapy in treating certain Th17-induced autoimmune diseases.

I.3.3.4 Regulatory T cells in the immune system

Regulatory T cells (Treg) play important roles in peripheral tolerance by inhibiting T cells over-reactive to self-antigens. The Tregs were first isolated and identified as $CD4^+CD25^+$ cells. However, activated $CD4^+$ T cells also express CD25. Later, FoxP3 was identified as the major marker of Treg. The Treg can be categorized into two subsets, the natural Tregs (nTreg) and the induced Tregs (iTreg), according to their origins [233].

The nTregs are generated in the thymic medulla [234]. nTreg development requires high-affinity interactions between TCR and self-peptide–MHC complexes presented by thymic stromal cells [235]. In addition, several factors have been identified to be important to support the development of Treg in the thymus. First, FoxP3, as a major marker of Tregs, is critical for T cells to differentiate to Treg [236]. Introducing FoxP3 gene into non Treg $CD4^+$ cells can endow the cells suppressive function in inhibiting the proliferation of other T cells [237]. Second, since the Tregs express the IL-2 receptor CD25, it is reasonable to address the role of IL-2 in Tregs. IL-2 is well known to be an important cytokine for T cell proliferation and differentiation. Mice lacking either CD25 or IL-2 showed a reduced number of Tregs. Similarly, mice with STAT-5 KO, a mediator of IL-2 signaling, also lose the Treg population [238, 239]. Moreover, new findings revealed that thymic stromal lymphopoietin (TSLP) generated by Hassall's corpuscles induces the generation of nTreg by activating thymic dendritic cells [240].

Apart from being generated in thymus, Tregs can also be generated from periphery by converting the naive $CD4^+$ T cells (e.g. iTreg). In the presence of TGF-β, naive $CD4^+$ T cells can be polarized into either Th17 or Treg. IL-2 is required to inhibit the Th17 potential and guarantee the conversion of $CD4^+$ naive T cell to Treg [241]. In addition, retinoic acid secreted by a specific subset of DCs is also reported to facilitate the Treg differentiation of CD4+ naive T cells by inhibiting the Th17 differentiation induced by IL-6 [242].

Treg requires activation by self-antigen exposure to exert its suppressive functions. Once activated, the Tregs' suppression function is independent of the antigen specificity. In vivo data show that the activated Tregs are capable of suppressing the proliferation and

differentiation of naive $CD4^+$ T cells. Moreover, other lymphocytes, such as $CD4^+$ T cells, $CD8^+$ T cells, NK cells as well as DCs can also be suppressed by Tregs [243]. The possible mechanisms include the secretion of immuno-regulatory cytokines, such as IL-10 and TGF-β, cell-cell contact inhibition, and modification and elimination of APCs. The roles of cytokines in immune suppression remain controversial based on contradictory data generated by in vitro and in vivo experiments. In vivo data from the IBD mouse model underline the importance of IL-10 in preventing colitis according to both IL10 KO and IL10 neutralization by Abs [244].

Interestingly, another study demonstrated that TGF-β1 bond to the cell surface of Treg executes the function in suppressing $CD4^+CD25^-$ cells in a cell-cell contact style [245]. Moreover, CTLA-4 has also been proposed to contribute to cell-cell contact suppression. CTLA-4 has high affinity to APC co-stimulator B7. It is possible that CTLA-4 expressed by Treg compete with CD28, which reduces the chance of activation of $CD4^+CD25^-$ cells by co-stimulation of TCR and CD28. In addition, CTLA-4 has been shown to induce the expression of IDO when binding to its ligands on DCs, which can potently suppress the ambient DCs. Consequently, Treg down-regulates the immune responses by suppressing the APCs [246].

When immune regulation is deficient, self-reactive lymphocytes and over-reactive lymphocytes are rendered uncontrolled, resulting in damage to normal tissues. In mouse models, it has been long-established that Treg deficiency will cause several autoimmune symptoms. Nude mice transferred with T cells without $CD25^+$ population develope autoimmune diseases while introducing back the $CD4^+CD25^+$ T cells can inhibit the symptoms [247]. Moreover, the mice lacking FoxP3 exhibit hyper-reactive $CD4^+$ cell and excessive IL-4, IL-6, IL-7, as well as TNF-α. Similar phenotypes are also observed in CTLA-4 and TGF-β deficient mice, indicating that a lack of Treg cells can cause autoimmune diseases [248]. In humans, deficiency of FoxP3 leads to a genetic disease referred as Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). The patients lack functional Tregs and suffer from severe autoimmune symptoms including autoimmune enteropathy, dermatitis, thyroiditis, and type 1 diabetes [249]. In another common autoimmune disease, RA, analysis of the Treg cells in RA patients revealed an interesting fact that the $CD4^+CD25^+$ Treg cells are actually enriched in the primary disease site, even in the

peripheral blood. However, the RA develops despite the presence of a high level of Treg. This may suggest that in RA patients the Treg are, at least, capable of responding to excessive immune responses and migrating to the disease site. However, the Treg failed to suppress the T cell activities [250]. Further studies showed that Tregs from RA patients have low level of FoxP3 expression which could explain the loss of function of patients' Tregs [251].

I.4 Working hypothesis of the project

Previous work of our group and others has reported the expression of Ephs and EFNs, particularly the B family members and some A family members, on thymocytes and T cells. These Ephs and EFNs function in modulating T cell responses and survival [88, 102, 252-257]. Our lab have demonstrated that EFNBs trigger Eph forward signalling and are capable of costimulating peripheral T cells in terms of enhancing cytokine production and proliferation in vitro [91-93]. To further investigate the roles of EFNBs in T cell development and function, we generated EFNB1, EFNB2 and EFNB1/2 knockout mice with T cell specific deletion.

On the other hand, our investigation into the roles of various EPHs and EFNs in the immune system disclosed that EPHB4 was expressed at a low level in mouse thymocytes, but at a high level in thymic stroma cells. It raised an interesting question: is thymic epithelial cell (TEC) EPHB4 expression essential in thymocyte development and, consequently, mature T-cell function in the periphery? Therefore, we generated conditional gene knockout (KO) mice with EPHB4 deletion in thymic epithelial cells (TECs).

The studies are presented and discussed in the following chapters.

II.Articles

II.1 Article 1

The effect of conditional EFNB1 deletion in the T cell compartment on T cell development and function

Wei Jin, Shijie Qi and Hongyu Luo

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Summary: In this study, we generated T-cell-specific EFNB1 gene knockout mice to further understand the role of EFNB1 in T cell immunity. We assessed T cell development and function in these mice. The mice were of normal size and cellularity in the thymus and spleen and had normal T cell subpopulations in these organs. The activation and proliferation of KO T cells was comparable to that of control mice. Naïve KO CD4 cells showed an ability to differentiate into Th1, Th2, Th17 and Treg cells similar to control CD4 cells. Our results suggest that the function of EFNB1 in the T cell compartment could be compensated by other members of the EFN family, and that such redundancy safeguards the pivotal roles of EFNB1 in T cell development and function.

WJ and HL participated in designing the experiments. WJ conducted all the experiments. WJ and HL participated in writing of the manuscript. All authors have read and approved the final version of the manuscript.

The effect of conditional EFNB1 deletion in the T cell compartment on T cell development and function

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Key words: EFNB1, conditional gene knockout, T cell development, T cell function

ABSTRACT

Background:

Eph kinases are the largest family of cell surface receptor tyrosine kinases. The ligands of Ephs, ephrins (EFNs), are also cell surface molecules. Ephs interact with EFNs transmitting signals in both directions, i.e., from Ephs to EFNs and from EFNs to Ephs. EFNB1 is known to be able to co-stimulate T cells *in vitro* and to modulate thymocyte development in a model of foetal thymus organ culture. To further understand the role of EFNB1 in T cell immunity, we generated T-cell-specific EFNB1 gene knockout mice to assess T cell development and function in these mice.

Results:

The mice were of normal size and cellularity in the thymus and spleen and had normal T cell subpopulations in these organs. The activation and proliferation of KO T cells was comparable to that of control mice. Naïve KO CD4 cells showed an ability to differentiate into Th1, Th2, Th17 and Treg cells similar to control CD4 cells.

Conclusions:

Our results suggest that the function of EFNB1 in the T cell compartment could be compensated by other members of the EFN family, and that such redundancy safeguards the pivotal roles of EFNB1 in T cell development and function.

II.1.1 BACKGROUND

Eph kinases are the largest family of cell surface receptor tyrosine kinases and can be divided into A and B subfamilies according to their sequence homology. The ligands of Ephs, ephrins (EFNs), are also cell surface molecules. EFNs are divided into A and B subfamilies. EFNAs are GPI-anchored cell surface proteins and EFNBs are transmembrane cell surface proteins. Ephs and EFNs interact promiscuously. EphA members mainly interact with EFNA members, while EphB members mainly interact with EFNB members. When Ephs and EFNs interact, signals are transmitted in both directions, i.e., from Ephs to EFNs and from EFNs to Ephs. Such bi-directional signalling is called forward and reversed signalling respectively (1).

EFNB1 is involved in the development and function of the central nervous system (2). It is also essential in bone maintenance and remodelling (3). EFNB1 expression in platelets contributes to the clotting process (4). Its expression on kidney epithelial cells (podocytes) likely plays a role in glomerular filtration (5). EFNB1 is involved in intestinal epithelial cell homeostasis (6). We have shown that EFNB1 forward signalling through their Eph receptors can costimulate peripheral T cells in terms of enhancing cytokine production and proliferation *in vitro* and enhance thymocyte survival (7,8). To further understand the role of EFNB1 in T cell development and function, we generated T-cell-specific gene knockout mice. The effects of EFNB1 on their immune system are presented here.

II.1.2 MATERIALS AND METHODS

Generation of T cell-specific EFNB1 gene knockout mice

T cell-specific EFNB1 gene knockout mice were generated in our laboratory. First, EFNB1 exon 1 was flanked with loxP sites. These floxed mice were backcrossed to the C57BL/6 background for more than nine generations and then mated with Lck-promoter-driven Cre transgenic (Tg) mice in the C57BL/6 background {strain B6.Cg-Tg(Lck-cre)I540Jxm/J, Jackson Laboratory, Bar Harbor, Maine, USA} to obtain T cell-specific EFNB1 gene knockout mice. Since EFNB1 is an X-linked gene, the deletion of an EFNB1 gene in one

allele in males equals the deletion of the gene in two alleles in females. These mice were collectively called EFNB1$^{f/f/cre}$ mice for convenience, regardless of gender. Floxed males or females without the Cre transgene were used as controls and were collectively called EFNB1$^{f/f}$ mice.

Immunofluorescent microscopy

One hundred thousand thymocytes in single cell suspension were applied to glass slides using Statspin® cytofuge (IRIS international INC., Westwood, MA, USA) at 850 RPM for 4 min. Cells were fixed with 4% paraformaldehyde for 15 min and then blocked with PBS containing 10% FBS. Cells were then stained with 4 μg/ml goat anti-EFNB1 Ab (Clone 94038, R&D systems, Minneapolis, MN, USA) overnight at 4□, followed by 0.25 μg/ml PE-donkey anti-goat IgG Ab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 45 min. The slides were mounted with prolong® Gold antifade reagent (Invitrogen, Eugene, OR) and examined under a Carl Zeiss microscope using AxioVision™ software (Jena, Germany).

Flow Cytometry

Single cell suspensions from the thymus and spleen were prepared and stained for flow cytometry as described in our previous publications (Yu et al., 2004; Yu et al., 2006). For intracellular antigen staining, cells were first stained with Abs against cell surface antigens fixed with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA), then stained with Ab against intracellular antigens. Next, Abs were used for flow cytometry as follows: anti-mouse CD4 PerCP (0.2μg/μl, Clone no. RM4-5); anti-mouse CD8 APC (0.2μg/μl, Clone no. 53-6.7); anti-mouse CD25 APC (0.2μg/μl, Clone no. 7D4); anti-mouse CD69 FITC (0.2μg/μl, Clone no. H1.2F3); anti-mouse Thy1.2 PE (0.2μg/μl, Clone no. 53-2.1); anti-mouse B220 APC-Cy™ 7 (0.2μg/μl, Clone no. RA3-6B2); anti-mouse IFN-γ FITC (0.2μg/μl, Clone no. B27); anti-mouse IL4 PE (0.2μg/μl, Clone no. BVD4-1D11); anti-mouse IL17 PE (0.2μg/μl, Clone no. TC11-18H10) (BD Bioscience Pharmingen, San Diego, CA, USA); and anti-mouse FoxP3 APC (0.2μg/μl, Clone no. FJK-16s) (eBioscience, San Diego, CA, USA).

T cell activation and proliferation assay

Total spleen cells were stimulated with soluble hamster anti-mouse CD3 mAb (100 ng/ml, clone 2C11) plus rat anti-mouse CD28 mAb (1μg/ml, clone 37.51.1). After 16 h culture, cells were stained with PE-rat anti-mouse Thy1.2 (clone no.53-21) and FITC-rat anti-CD25 (clone 7D4) or anti-CD69 mAb (clone H1.2F3), and analyzed with 2-color flow cytometry. For T cell proliferation, spleen T cells were purified using Easysep® Mouse T cell enrichment kit (Stemcell technologies, Vancouver, BC, Canada) and cultured in wells coated with hamster anti-mouse CD3 mAb (1μg/ml for coating) alone or hamster anti-mouse CD3 mAb (0.1μg/ml for coating) plus rat anti-mouse CD28 (1μg/ml for coating). ^{3}H-thymidine uptake of the cultured cells was measured at 24 h, 48 h and 72 h after culture, as described in our previous publication (7).

In vitro *Th1, Th2, Th17 and Treg polarization*

Th1, Th2, Th17 and Treg populations were polarized from naïve $CD4^+$ T cells that were isolated from pooled splenocytes and lymph node cells using Naïve $CD4^+$ T Cells Isolation Kits (R&D Systems). Purity of the naïve $CD4^+$ cells was routinely greater than 95%. Purified naïve T cells (0.1×10^6/well) were mixed with T cell-depleted irradiated (3000 Rads) C57BL/6 feeder splenocytes (0.5×10^6 cells/well), and cultured in 96-well plates in RPMI medium 1640 containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin G, 1× nonessential amino acids, 1 μM sodium pyruvate, 2.5 μM β-mercaptoethanol, and 2 μg/ml soluble hamster anti-CD3ε mAb (clone 145-2C11; 2 μg/ml). For Th1 polarization, rmIL-12 (10 ng/ml) and rat anti-IL-4 mAb (10 μg/ml, clone 11B11) were added to the culture. For Th2 polarization, rmIL-4 (20 ng/ml), rat anti-IL-12 mAb (Clone no.48110 10 μg/ml,) and rat anti-IFN-γ mAb (10 μg/ml, Clone no 37895) were added. For Th17 polarization, cultures were supplemented with recombinant mouse IL-6 (20 ng/ml), recombinant human TGF-β1 (5 ng/ml), and rat anti-IL-4 mAb and rat-anti-IFN-γ mAb (10 μg/ml). For Treg polarization, recombinant human TGF-β1 (5 ng/ml) and rat anti-IL-4 and rat anti-IFN-γ mAb (both at 10 μg/ml) were added to the culture. Recombinant cytokines and mAb against cytokines were all from R & D Systems (Minneapolis, MN, USA). Five days after culture, 5 nM of PMA, 500 ng/ml of ionomycin, and protein transport inhibitor BD GolgiStop™ (BD Bioscience; San Diego, CA, USA) were

added for the last four hours of culture. Cells were harvested and stained for intracellular cytokines or FoxP3 followed by flow cytometry analysis.

II.1.3 RESULTS

EFNB1 deletion in T cells

To confirm the deletion of EFNB1 in the T cell compartment, we conducted an immunofluorescence study on thymocytes using Thy1.2 and EFNB1 double staining. Control Thy1.2^{+} thymocytes (in green) expressed EFNB1 (in red) (Fig. 1, upper row), while EFNB1 was not detectable in Thy1.2^{+} thymocytes from EFNB1$^{f/f/cre}$ mice (Fig. 1, bottom row). This demonstrated that EFNB1 was indeed deleted in the T-linage cells in KO mice.

Cellularity and cell subpopulations in the thymus and spleen of* EFNB1$^{f/f/cre}$ *mice

There was no gross morphological difference between EFNB1$^{f/f/cre}$ and EFNB1$^{f/f}$ thymi. They were of similar weight and cellularity, as shown in Figures 2. Thymocytes from EFNB1$^{f/f/cre}$ mice were analyzed by flow cytometry for different cell populations. Representative histograms are shown in Figure 2B, and bar graphs in Figure 2C summarize the results of 13 independent experiments. EFNB1$^{f/f/cre}$ and EFNB1$^{f/f}$ mice had comparable percentages of CD4CD8 double positive (DP), CD4 single positive (SP), and CD8 SP thymocytes. Conversely, there was a moderate but significant increase in CD4CD8 double negative thymocytes in EFNB1$^{f/f/cre}$ mice compared to EFNB1$^{f/f}$ mice.

The spleen weight and cellularity of EFNB1$^{f/f/cre}$ mice showed no significant difference compared to controls (Figs. 3A & 3B) after analyzing 13 pairs of mice. Different spleen cell populations were assessed by flow cytometry. Histograms from representative experiments are shown in Figures 3C and 3D, and a summary of results from 13 independent experiments are illustrated as bar graphs in Figure 3E. T and B cell populations in the EFNB1$^{f/f/cre}$ spleens were comparable to those of EFNB1$^{f/f}$ spleens (Figs. 3C & 3E). No apparent difference was found in the ratios of CD4 and CD8 cells between KO and control spleens (Figs. 3D & 3E). Results show that T cell development was minimally affected in the absence of EFNB1.

Activation and proliferation of* EFNB1$^{f/f/cre}$ *T cells were not compromised

Next, we investigated the function of peripheral EFNB1$^{f/f/cre}$ T cells in terms of activation and proliferation. Spleen T cells were purified by negative selection using magnetic beads; the purified cells contained more than 95% CD3 positive cells. They were cultured in wells coated with anti-CD3 and anti-CD28. After overnight culture, they were analyzed for the expression of activation markers such as CD25 and CD69. More than 95% of the T cells from EFNB1$^{f/f/cre}$ mice upregulated their CD25 and CD69 expression within 16 h; such upregulation was comparable to that of T cells from EFNB1$^{f/f}$ mice (Fig. 4A).

We then examined proliferation of T cells from EFNB1$^{f/f/cre}$ mice. Purified spleen T cells were cultured in wells coated with anti-CD3 mAb (1 μg/ml for coating), or anti-CD3 plus anti-CD28 mAb (0.1 μg/ml and 1 μg/ml for coating, respectively); and their proliferation was determined at 24, 48 and 72 h according to ^{3}H-thymidine uptake. A summary of three independent experiments is illustrated in Figures 4B and 4C. Overall, EFNB1$^{f/f/cre}$ T cells showed no compromise in their ability to proliferate upon solid phase anti-CD3 mAb (Fig. 4B) or anti-CD3 plus anti-CD28 mAb (Fig. 4C) stimulation. Of importance, anti-CD3 Ab concentration (1 μg/ml) was higher in wells coated with anti-CD3 Ab alone, compared to the concentration used in wells coated with anti-CD3 plus anti-CD28 Abs. This shows that the maximal proliferation under these two conditions was similar.

In vitro differentiation of EFNB1$^{f/f/Cre}$ T cells

Since EFNB1$^{f/f/Cre}$ T cells showed no abnormality in activation and proliferation, we next assessed whether they could properly differentiate into different functional subpopulations. Spleen naive CD4 T cells from EFNB1$^{f/f/Cre}$ and control EFNB1$^{f/f}$ mice were cultured under conditions favouring the development of Th1 (Fig. 5A), Th2 (Fig. 5B), Th17 (Fig. 5C) and Treg (Fig. 5D) cells. After optimal durations of culture for each type of cell, the cells were collected and analyzed for their intracellular IFN-γ, IL4, IL17 and FoxP3 content by flow cytometry. After differentiation, as shown in Figures 5A-5D, EFNB1$^{f/f/Cre}$ and EFNB1$^{f/f}$ CD4 cells were comparable in their percentage of intracellular IFN-γ^{+}, IL4^{+}, IL17^{+} and FoxP3^{+} cells.

This indicates that EFNB1$^{f/f/Cre}$ CD4 cells are capable of normal differentiation into Th1, Th2, Th17 and Treg cells.

II.1.4 DISCUSSION

We investigated T cell development in mice with T cell-specific deletion of EFNB1. Results show that in the absence of EFNB1, the thymus and spleen showed mostly normal subpopulations of T cell origin. Moreover, mature T cells had no apparent defects in their activation and proliferation or in their ability to differentiate into functional Th1, Th2, Th17 and Treg cells.

Previous *in vitro* studies using foetal thymus organ culture have shown that recombinant EFNB1 protein is capable of influencing T cell development and enhancing thymocyte survival (8). In another study (7), solid phase EFNB1 was shown to increase mature T cell response to TCR stimulation. How do we reconcile these observations with the present findings that EFNB1 deletion showed not apparent impact on T cell development and mature T cell function? As mentioned in the introduction, EFNB1 promiscuously interacts with multiple EphB family members such as EphB1, B2, B3, B4, and B6, as well as with a few EphA family members such as EphA4 and EphA3. When soluble EFNB1 is introduced into thymus organ culture, it not only blocks the interaction of multiple Ephs with EFNB1, but also likely interferes with interactions between those Ephs and other EFN members. When EFNB1 is placed on solid phase, it will noticeably trigger signalling of multiple Ephs. Such effects cannot be achieved by simple EFNB1 deletion. In the absence of EFNB1, its forward signalling to other Eph molecules and its reverse signalling are likely compensated by other EFNs.

The absence of a demonstrated phenotype in T cell development and function following EFNB1 deletion does not prove that this molecule is unimportant. On the contrary, this observation suggests an essential importance of EFNB1. It can be argued that the role of EFNB1 in the T cell compartment is so essential, that any accidental mutation could lead to disastrous consequences. As a counter measure to prevent such disasters from occurring,

through evolution, we have developed highly redundant Eph and EFN systems, as well as promiscuous interactions between Eph and EFN members. With such a system, any aberrant mutation that occurs to certain Eph or EFN members will be safely compensated by others in such a way as to guarantee their normal function in the T cell compartment. It is further inferred that the true importance of EFNs can only be revealed if a deletion of multiple EFN family members occurs at the same time. Through our studies on T cell-specific deletion of both EFNB1 and EFNB2 in mice (data to be published elsewhere), we found that in the absence of both EFNB1 and EFNB2, there was compromised αβT cell development in the thymus along with abnormal thymic structure. The EFNB1/EFNB2 KO T cells significantly failed to compete with normal T cells during homeostatic expansion in irradiated recipients. EFNB1/EFNB2 double KO T cells were significantly inferior to normal T cells in their ability to differentiate into Th1 and Th17 cells. Such a handicap resulted in compromised *in vivo* T cell-mediated immune responses such as allograft rejection and anti-virus immunity. These results confirm the critical role of EFNB1 in T cell development and function. However, this role was not observed in the EFNB1 single KO mice used in this study.

II.1.5 CONCLUSIONS

To conclude, we are the first to have carried out an analysis of roles of T cell development and function in the mice with conditional EFNB1 deletion in the T cell compartment, though the effect of *the single EFNB1 KO* appears to be minimal. Our results suggest that the function of EFNB1 in the T cell compartment could be compensated by other members of the EFN family, and that such redundancy safeguards the pivotal roles of EFNB in T cell immunity. Additional studies on EFNB family, i.e. EFNB1, EFNB2 and EFNB3, double or even triple null mutated mice to investigate T cell development and function are warranted to confirm such indications.

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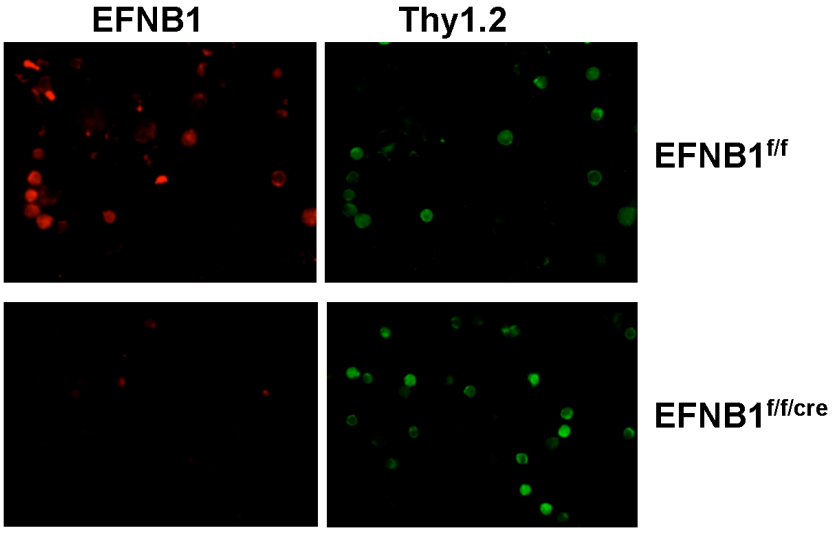
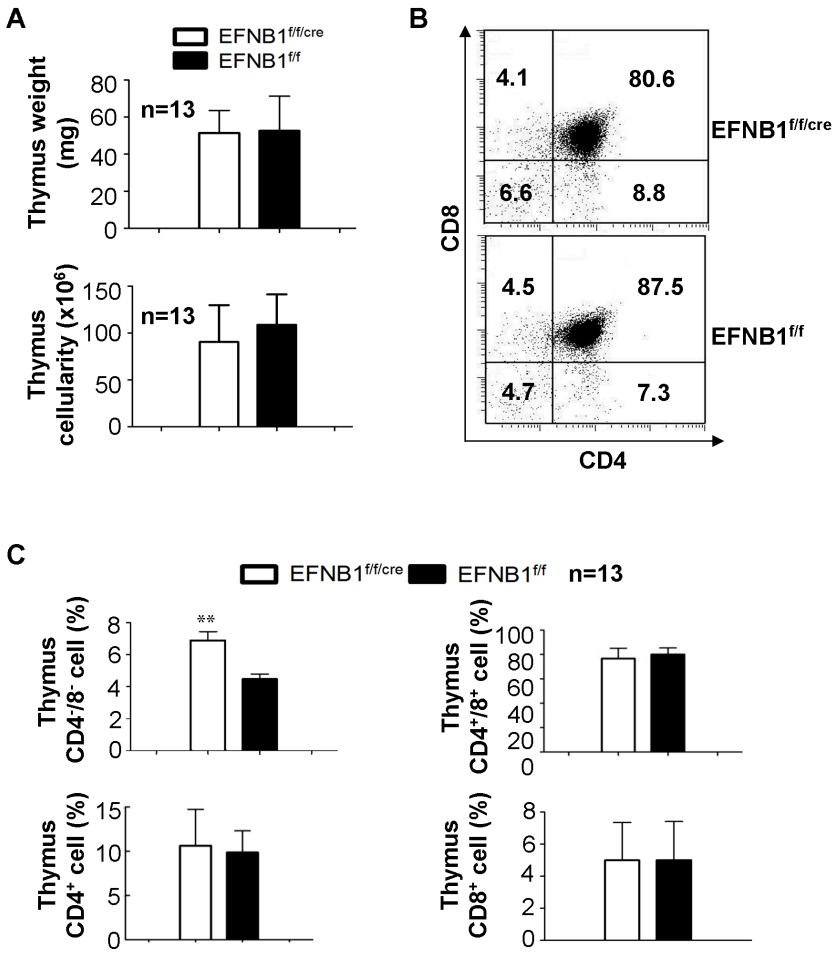
II.1.8 FIGUREs and LEGENDS

Figure 2.1. T cell-specific deletion of EFNB1 in Lck-EFNB1$^{f/f}$ mice according to immunofluorescent microscopy



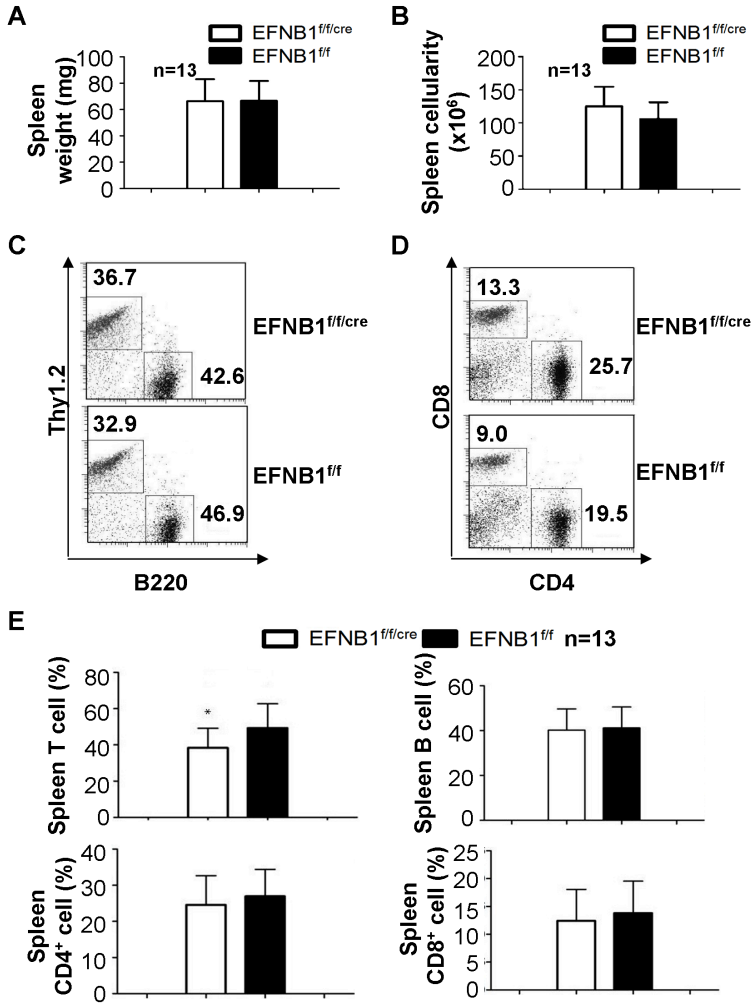
Thymocytes from EFNB1$^{f/f}$ (upper row) or Lck-EFNB1$^{f/f}$ (bottom row) mice were stained with FITC-rat anti-Thy1.2 (green) and PE-rat anti-EFNB1 (red) mAbs. Micrographs of the cells with pseudocolouring are shown.

Figure 2.2. Phenotype of Lck-EFNB1$^{f/f}$ thymus



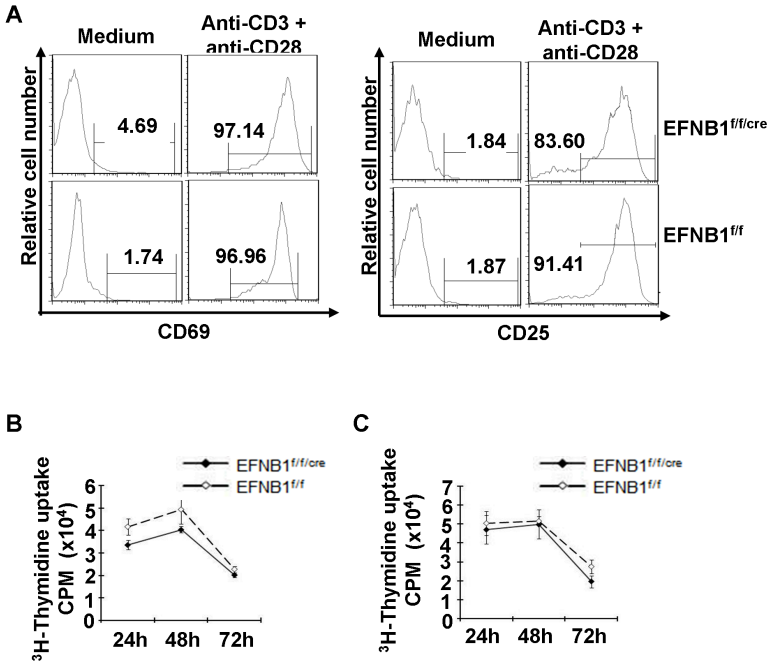
A. Thymus size and cellularity of Lck-EFNB1$^{f/f}$ mice N=13 pairs ($p>0.05$; paired Student's t test). B and C. Subpopulations of thymocytes from Lck-EFNB1$^{f/f}$ and EFNB1$^{f/f}$ mice according to flow cytometry Representative histograms are shown in B and bar graphs summarize data from 13 independent experiments are illustrated in C ($p>0.05$; paired Student's t test).

Figure 2.3. Phenotype of Lck-EFNB1$^{f/f}$ spleens



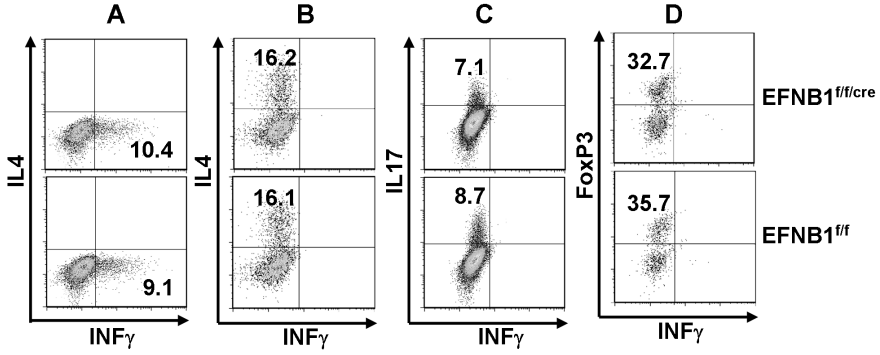
A and B. Spleen size and cellularity of Lck-EFNB1$^{f/f}$ and EFNB1$^{f/f}$ mice N=13 pairs ($p > 0.05$; paired Student's t test). C and D. Spleen T cell, B cell, CD4 T cell and CD8 T cell population of Lck-EFNB1$^{f/f}$ mice according to flow cytometry. Representative histograms are shown in C and D, and bar graphs summarize data from 13 independent experiments are illustrated in E ($p>0.05$; paired Student's t test).

Figure 2.4. Normal activation and proliferation of Lck-EFNB1$^{f/f}$ T cells



A. Activation marker expression Total spleen cells from *Lck-EFNB1$^{f/f}$ and EFNB1$^{f/f}$* mice were stimulated with soluble anti-CD3 mAb and anti-CD28 mAbs for 48 hours, and stained with PE-rat anti-mouse Thy1.2 and FITC-rat anti-mouse CD25 or CD69, followed by flow cytometry analysis. B and C. T cell proliferation Purified T cells from *Lck-EFNB1$^{f/f}$ and EFNB1$^{f/f}$* mice were cultured in wells coated with solid phase anti-CD3 (B) or anti-CD3 plus anti-CD28 mAb (C) for 24 to 72 h. The cells were pulsed with ^{3}H-thymidine for the last 16 h of the culture. Samples were in triplicate. Mean ± SD of cpm are shown. Experiments were repeated more than 5 times and representative data are shown.

Figure 2.5. CD4 cell in vitro differentiation



Naïve CD4 cells were cultured under conditions favouring Th1 (A), Th2 (B), Th17 (C) and Treg (D) cells. Their intracellular cytokine or FoxP3 expression was determined by flow cytometry. Experiments were repeated more than 5 times and representative data are shown.

II.2 Article 2.

T cell-specific deletion of EFNB2 minimally affects T cell development and function

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Mol Immunol. 2012 Oct;52(3-4):141-7

Summary: In this study, using mice with T cell-specific EFNB2 gene knockout (EFNB2 KO mice), we investigated T cell development and function after EFNB2 deletion. Our results suggest the involvement of EFNB2 in thymocyte development. However, heavy redundancy among Eph/EFN family members prevents the occurrence of detrimental phenotypes in the T cell compartment caused by T cell-specific EFNB2 gene null mutation.

WJ and HL participated in designing the experiments. WJ conducted all the experiments. WJ and HL participated in writing of the manuscript. All authors have read and approved the final version of the manuscript.

T cell-specific deletion of EFNB2 minimally affects T cell development and function

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Running title: the role of EFNB2 in T cell development and function

Key words: EFNB2, conditional gene knockout, T cell development, T cell function

ABSTRACT

Background:

Eph kinases and their ephrin ligands (EFN) are all cell surface molecules, capable of transmitting signals in both directions (1, 2). Such bidirectional signaling is called forward (from EFNs to Ephs) and reverse (from Ephs to EFNs) signaling. Eph family kinases have 15 members, divided into A and B subfamilies. Ephrin ligands have 9 members, also classified into A and B families. Ephs and ephrins interact promiscuously, but EphAs mainly interact with EFNAs, and EphBs with EFNBs. EphB family kinases and their ephrin ligands (EFN) are expressed in the T cell compartment.

Results:

In this study, using mice with T cell-specific EFNB2 gene knockout (EFNB2 KO mice), we investigated T cell development and function after EFNB2 deletion. EFNB2 KO mice presented normal thymus weight and cellularity. Their thymocyte subpopulations, such as CD4CD8 double positive cells and CD4 and CD8 single positive cells, were normally distributed, but there was a significant relative increase of CD4CD8 double negative cells. Flow cytometry analysis revealed that there was a moderate increase in the DN3 subpopulation. This augmented percentage of DN cells was further confirmed in competitive repopulation chimeras, suggesting that EFNB2 is involved in thymocyte development. The EFNB2 KO mice had normal T cell numbers and percentages in the spleen, and the T cells were able to be activated and to proliferate normally upon TCR ligation. Further, EFNB2 KO naïve CD4 cells were capable of differentiating into Th1, Th2, Th17 and Treg cells similar to WT naïve CD4 cells.

Conclusions:

Our results suggest the involvement of EFNB2 in thymocyte development. However, heavy redundancy among Eph/EFN family members prevents the occurrence of detrimental phenotypes in the T cell compartment caused by T cell-specific EFNB2 gene null mutation.

II.2.1 BACKGROUND

Eph kinases and their ephrin ligands (EFN) are all cell surface molecules, capable of transmitting signals in both directions (1,2). Such bidirectional signaling is called forward (from EFNs to Ephs) and reverse (from Ephs to EFNs) signaling. Eph family kinases have 15 members, divided into A and B subfamilies. Ephrin ligands have 9 members, also classified into A and B families. Ephs and ephrins interact promiscuously, but EphAs mainly interact with EFNAs, and EphBs with EFNBs (1-3).

Functions of Ephs were initially discovered in the central nerve system, where they control axon and dendrite positioning (2,3). They are essential in the development of neuronal connections, circuit plasticity and repair. However, in recent years, additional functions of Ephs and EFNs have been found in various tissues and organs. They are vital in angiogenesis during normal embryonic development as well as in tumorigenesis (4,5). Some Eph and EFNB family members modulate epithelium self-renewal in the intestine (6). EphB2 and EFNB2 bidirectional interaction is essential in urorectal development (7). Pancreatic β-cells communicate with each other via EphA and EFNA family members to synchronize their insulin secretion in response to blood glucose fluctuations (8). Several EphB and EFNB family members regulate bone development, maintenance and repair (1, 9, 10). Multiple Eph and EFN members seem to influence cancer cell growth (5, 11). EphB4 and EFNB2 regulate red blood cell production in response to hypoxia (1). EFNB1 and EphA4 contribute to the clotting process (2). EFNB1 likely plays a role in glomerular filtration (12). The interaction between EphB2 and EFNB2 regulates the ionic homeostasis of vestibular endolymph fluid in the inner ear (13).

We have previously shown that EphB6, although lacking kinase activity, can transmit signals into T cells, and that its null mutation leads to compromised T cell response *in vitro* and *in vivo* (14). Forward signaling from EFNB2 to Eph functions as costimulation signals in T cells to enhance cytokine production and proliferation *in vitro* (15). In this study, we generated

gene knockout mice with T cell-specific EFNB2 deletion and studied the role of EFNB2 in T cell development and function in these mice.

II.2.2 MATERIALS AND METHODS

Generation of T cell-specific EFNB2 gene knockout mice

T cell-specific EFNB2 gene knockout mice were generated in our laboratory. First, exon 1 of the EFNB2 genomic sequence was flanked with loxP sites in the targeting plasmid construct, and the construct was transfected into embryonic stem (ES) cells. After selection, the ES cells were used to generate chimera mice and then germ line transmission of the floxed EFNB2 gene. These floxed mice were backcrossed to the C57BL/6 background for more than eight generations and then mated with Lck-promoter-driven Cre transgenic (Tg) mice in the C57BL/6 background (strain B6.Cg-Tg(Lck-cre)I540Jxm/J, Jackson Laboratory, Bar Harbor, Maine, USA) to obtain T cell-specific EFNB2 gene knockout mice. All animal protocols were approved by the Institutional Animal Protection Committee of the University of Montreal Hospital Center (N09055JWs).

Immunofluorescent microscopy

One hundred thousand thymocytes in single cell suspension were applied to glass slides using Statspin® cytofuge (IRIS international INC., Westwood, MA, USA) at 850 RPM for 4 min. Cells were fixed with 4% paraformaldehyde for 15 min and then blocked with PBS containing 10% FBS. Cells were then stained with 4 μg/ml goat anti-mouse EFNB2 Ab (R&D systems, Minneapolis, MN, USA) overnight at 4□, followed by 0.25 μg/ml PE-donkey anti-goat IgG Ab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 45 min. The slides were mounted with prolong® Gold anti-fade reagent (Invitrogen, Eugene, OR) and examined under a Carl Zeiss microscope using AxioVision™ software (Jena, Germany).

Reverse transcription/quantitative polymerase chain reaction (RT/qPCR)

EFNb2 mRNA levels were measured by RT/qPCR. Total RNA from cells was extracted using TRIzol® (Invitrogen, Carlsbad, CA) and then reverse-transcribed with Superscript II™ reverse-transcriptase (Invitrogen). Primers used are 5'-

CCCTTTGTGAAGCCAAATCCAGGT-3' (forward primer) and 5'-TCCTGATGCGATCCCTGCGAATAA-3' (reverse primer). qPCR condition for the reaction was as follows: two minutes at 50^0C, two minutes at 95^0C followed by 45 cycles of 10 seconds at 94^0C, 20 seconds at 58^0C and 20 seconds at 72^0C. β-actin mRNA level was used as internal controls and data were expressed as signal ratios of EFNb2 gene mRNA/β-actin gene mRNA.

Flow Cytometry

Single cell suspensions from the thymus and spleen were prepared and stained for flow cytometry as described in our previous publication (15, 16). DN stains were performed on lineage-negative thymocytes using anti-CD25 (clone PC61) and anti-CD44 (clone 1M7) Abs. Lineage-negative cells were identified using a mixture of anti-CD3e (clone 145-2C11), anti-TCRβ (clone H57-597), anti-B220 (clone RA3-6B2), anti-CD11b (clone M1/70), anti-TCRγδ (clone GL-3), anti-NK1.1 (clone PK136), anti-CD8a (clone 53-6.7), anti-Ter119 (clone Ter119)，and anti-Gr-1 (clone RB6-8C5) Abs to gate out all those lineage positive cells. For intracellular antigen staining, cells were first stained with Abs against cell surface antigens fixed with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA), then stained with Ab against intracellular antigens. Abs used for flow cytometry were as follows: PerCP-conjugated rat anti-mouse CD4 mAb (clone RM4-5); APC-conjugated rat anti-mouse CD8 mAb (clone 53-6.7); APC or PE-conjugated rat anti-mouse CD25 mAb (clone PC61); FITC-conjugated rat anti-mouse CD69 mAb (clone H1.2F3); PE-conjugated rat anti-mouse Thy1.2 mAb (clone 53-2.1); APC-Cy^{TM} 7-conjugated rat anti-mouse B220 mAb (clone RA3-6B2); FITC-conjugated mouse anti-mouse CD45.1 (clone A20); PerCP-conjugated mouse anti-mouse CD45.2 (clone 104); Pacific blue-conjugated rat anti-mouse CD44 (clone 1M7); APC-conjugated rat anti-mouse c-Kit (clone 2B8); FITC-conjugated rat anti-mouse IFN-γ mAb (clone XMG1.2); PE-conjugated rat anti-mouse IL-4 mAb (clone BVD4-1D11); PE-conjugated rat anti-mouse IL-17 mAb (clone TC11-18H10) and APC-conjugated rat anti-mouse FoxP3 mAb (clone FJK-16S). These Abs were purchased from BD Bioscience Pharmingen , BioLegend, and eBioscience (San Diego, CA, USA).

Generation of bone marrow chimeras

Eight to 10-week-old C57BL/6 ($CD45.2^+$) x C57/B6.SJL($CD45.1^+$) F1 mice were irradiated at 1,100 Rads. Twenty-four hours later, they received $4x10^6$ T cell-depleted bone marrow cells from $EFNb1^{f//f/cre}$ and C57/B6.SJL mice in a 1:1 ratio. $EFNb1^{f//f}$ mouse bone marrow was used as a control. Thymocytes and spleen cells of the recipients were analyzed by flow cytometry 8 to 10 weeks following the bone marrow transplantation (BMTx).

T cell activation and proliferation assay

Total spleen cells were stimulated with soluble hamster anti-mouse CD3 mAb (clone 2C11; BD Bioscience; San Diego, CA, USA; 100 ng/ml) plus rat anti-mouse CD28 mAb (clone 37.51.1; Cedarlane, Canada; 1μg/ml). After 16 h-culture, cells were analyzed with 2-color flow cytometry for CD69 and CD25 expression. For T cell proliferation, spleen T cells were purified using an Easysep® Mouse T cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) and cultured in wells coated with hamster anti-mouse CD3 mAb (1μg/ml for coating) alone or with hamster anti-mouse CD3 mAb (0.1μg/ml for coating) plus rat anti-mouse CD28 (1μg/ml for coating). ^{3}H-thymidine uptake of the cultured cells was measured at 24 h, 48 h and 72 h after culture with ^{3}H-thymidine added to the culture 16 h before the harvest, as described in our previous publication (17).

In vitro Th1, Th2, Th17 and Treg polarization

Th1, Th2, Th17 and Treg populations were polarized from naïve $CD4^+$ T cells that were isolated from pooled splenocytes and mesenteric lymph node cells using Naïve $CD4^+$ T Cell Isolation Kits (R&D Systems). Purity of the naïve $CD4^+$ cells was routinely greater than 95%. These CD4 cells (0.25×10^6/well) were mixed with feeder cells (1.25×10^6 cells/well), which were T cell-depleted C57BL/6 splenocytes after 3000 Rads irradiation, and cultured in 96-well plates in RPMI medium 1640 containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin G, 1× nonessential amino acids, 1 μM sodium pyruvate, 2.5 μM β-mercaptoethanol, and 2 μg/ml soluble hamster anti-CD3ε mAb (clone 145-2C11; 2 μg/ml). For Th1 polarization, mouse IL-12 (10 ng/ml) and rat anti-IL-4 mAb (clone 11B11; 10 μg/ml) were

added to the culture. For Th2 polarization, mouse IL-4 (20 ng/ml), rat anti-mouse IL-12 mAb (clone 48110; 10 μg/ml) and rat anti-IFN-γ mAb (clone 37895; 10 μg/ml) were added. For Th17 polarization, cultures were supplemented with mouse IL-6 (20 ng/ml), human TGF-β1 (5 ng/ml), and rat anti-mouse IL-4 mAb (clone 30340, concentration) and rat anti-mouse IFN-γ mAb (clone 37895; 10 μg/ml). For Treg polarization, human TGF-β1 (5 ng/ml) and rat anti-mouse IL-4 mAb and rat anti-mouse IFN-γ mAb (both at 10 μg/ml) were added to the culture. Recombinant cytokines and mAbs against cytokines were all from R & D Systems (Minneapolis, MN, USA). Three days (for Th1 and Th17) or 5 days (for Th2) after culture, 5 nM of PMA, 500 ng/ml of ionomycin, and protein transport inhibitor BD GolgiStop™ (BD Bioscience; San Diego, CA, USA) were added for the last four hours of culture. Cells were harvested and stained for intracellular cytokines followed by flow cytometry analysis.. . Naive CD4+ T cells under Treg culture condition were subject to intracellular staining for FoxP3 directly when harvested after three-day culture.

II.2.3 RESULTS

EFNB2 null mutation is embryonic lethal. To investigate the role of EFNB2 in the T cell compartment, we generated T cell-specific gene knockout mice using the loxP/Cre recombinase system. The floxed mice were backcrossed with C57BL/6 mice for more than 8 generations and were then mated with Tg mice with Cre recombinase expression driven by a Lck promoter to achieve T cell-specific deletion of EFNB2. These T-cell-specific EFNB2 null mutant mice were called KO mice, and the floxed mice without Cre transgene were called WT mice for the sake of simplicity. The deletion of EFNB2 in thymocytes and spleen T cells at the protein level was confirmed by immunofluorescent microscopy and flow cytometry. As shown in Figure 1, EFNB2 was detectable in WT but not KO Thy1.2^{+} thymocytes (A) and spleen T cells (B). RT-qPCR further corroborated EFNB2 deletion in spleen CD4 and CD8 cells (C).

The T cell-specific EFNB2 KO mice were fertile and showed no discernable anomaly on visual inspection. Their thymus weight and cellularity were comparable to those of WT mice (Figs. 2A and 2B). We next assessed the percentage of thymocyte subpopulations by flow cytometry. A representative histogram was shown in Fig. 2C, and the bar graphs in Fig. 2D

summarize data from 10 pairs of KO and WT mice. The percentages of CD4CD8 that were double positive (DP), CD4 single positive (SP), and CD8 SP cells in the KO thymus were similar to those in the WT thymus. All CD4 and CD8 SP thymocyted in KO mice were CD3 positive as in WT mice (data not shown). However, there was a moderate but statistically significant increase in the percentage of CD4CD8 double negative (DN) cells in the KO thymuses compared to WT thymuses, suggesting the involvement of EFNB2 in early T cell development. We then evaluated different DN subpopulations by staining the DN cells with anti-CD25 and anti-CD44 antibodies. As shown in Fig. 2E, there was a small but significant augumentation of DN3 stage cells in EFNB2 KO thymocyte, indicating that EFNB2 might play a role in the proper progress of thymocytes from the DN3 to DN4 stages.

Next, we compared subpopulations of DN2s and DN3s in WT and EFNB2 KO thymocytes by their c-Kit and icTCRβ expression. As shown in Fig. 3, no apparent difference in the proportion of c-Kit^{high} (DN2a), c-Kit^{low} (DN2b), icTCRβ^{-} (DN3a) or icTCRβ^{+} (DN3b) was observed between EFNB2 KO and control WT thymi. The similar percentage of icTCRβ positive cells in DN3 stage implies that the slight increase of DN3 in EFNB2 KO does not seem to be the result of unsuccessful rearrangement of TCR-β chain.

In the peripheral immune system, the spleen weight and cellularity of the KO mice showed no significant difference from those of WT mice (Figs. 4A and 4B). The T cell versus B cell percentage and CD4 cell versus CD8 cell percentage in the KO spleen were analyzed by flow cytometry. A representative histogram was illustrated in Figs. 4C and 4D, and data for 10 pairs of KO and WT mice are presented as bar graphs in Fig. 4E. The result demonstrates that there is no significant difference between KO and WT mice in the spleen T cell and B cell percentage and in the CD4 and CD8 T cell subpopulations.

The subtle but statistically significant increase of KO thymocytes prompted us to further examine thymopoiesis from KO bone marrow progenitors in competitive repopulation experiments. We injected mixtures of equal numbers of T-cell depleted bone marrow cells from EFNB2$^{f/f/cre}$ (CD45.2^{+}) mice and competitor B6.SJL (CD45.1^{+}) mice into lethally irradiated heterozygous B6xB6.SJL F1 (CD45.1^{+}CD45.2^{+}) mice. EFNB2$^{f/f}$ mice were used as wild type control. As shown in Fig. 5A, bone marrow progenitor from donor EFNB2 KO mice

and competitor B6.SJL mice contributed almost equally to the reconstituted pool of thymocytes. No difference was noted when compared with bone marrow progenitor from donor EFNB2 WT mice and competitor B6.SJL mice (left panel). However, increased percentage of lineage negative cells was found in CD45.2 single positive cells of KO mice compared to that of WT mice (right panel). -These data further supported the role of EFNB2 during thymocyte development.

We also analyzed reconstituted spleen cells in these mixed chimeras (Fig 5B). Both KO and WT donor cells repopulated host spleen to similar extents, and no competitive advantage or disadvantage of KO or WT progenitors were observed when competing with B6.SJL progenitors (left panel). Comparing to WT bone marrow progenitors, KO progenitors generated the same average number of CD3 positive T cells (right panel). The results in these competitive repopulation experiments were consistent with the observation in EFNB2 KO mice.

We then investigated the function of KO T cells. The expression of early activation marker CD69 and the upregulation of CD25 in KO and WT T cells upon anti-CD3 plus anti-CD28 stimulation showed no significant differences (Figs. 6A and 6B). The proliferative response of KO and WT T cells upon soluble anti-CD3 Ab or anti-CD3 Ab plus anti-CD28 Ab stimulation was comparable, as shown in Figures 6C and 6D, respectively.

The in vitro differentiation of naïve CD4 cells from KO and WT mice was also assessed. Spleen and lymph node naïve CD4 cells were isolated from KO and WT mice and cultured in conditions favoring the development of Th1, Th2, Th17 or Treg. After 3 or 5 days as each condition required, the CD4 cells were stained for intracellular cytokines (IFNγ, IL-4 or IL-17) or FoxP3, and analyzed with 3-color flow cytometry. As shown in Fig. 7, KO and WT naïve CD4 cells presented comparable capability to differentiate into Th1, Th2, Th17 or Treg cells.

II.2.4 DISCUSSION

EphB family and their ligands are expressed in the immune cells. Data from our laboratory (not shown) indicate that all five EphB family members and 3 EFNB family members are expressed on thymocytes and mature T cells, suggesting that they are involved in T cell development and/or function. Our previous publication (15) using recombinant EFNB2 in an in vitro system showed that forward signaling from EFNB2 to Eph provides T cells with costimulation. However, in this study, the T cell specific deletion of EFNB2 seems to have minimally affected T cell development and function, with the exception of a relative increase in DN cell population in the EFNB2 KO thymus. Considering the promiscuous interaction between EFN and Eph and the multiple members of the Eph and EFN families, this is probably understandable. It is very likely that the missing function of EFNB2 in the conditional KO mice is compensated by other members of the EFN family, particularly the members of the EFNB subfamily. Our previous in vitro experiments using solid phase EFNB2 showed costimulation. This could be due to the fact that the solid phase EFNB2 acts on multiple Ephs, which collectively elicit stronger responses.

It follows that if the redundancy is removed by a deletion of multiple EFNBs in the T cell compartment at the same time, the true effect of EFNB2 in this regard will be revealed. This is indeed the case, based on our recent findings in T cell-specific EFNB1 and EFNB2 double KO mice. In these mice, there was abnormal thymus structure accompanied by significantly reduced thymus size, weight and cellularity. The percentage of DN thymocytes in the double KO mice was abnormally increased, with a higher percentage than that in the EFNB2 single KO mice, confirming that the relative increase of DN percentage in the EFNB2 KO thymus is a valid phenotype. Spleen size and cellularity along with T cell number in the EFNB1/EFNB2 double KO mice were drastically reduced. In vitro and in vivo T cell functions, such as Th1 and Th17 differentiation, allograft rejection and anti-viral immune responses, were all compromised in these mice (18).

II.2.5 CONCLUSIONS

To conclude, we have examined the roles of EFNB2 in T cell development and function in T cell-specific EFNB2 gene knockout mice. Our current study demonstrates that T cell-specific deletion of EFNB2 minimally affects T cell development and function.

II.2.6 AUTHORS' CONTRIBUTIONS

All authors participated in experimental procedures leading to figures contained within. WJ and HL participated in writing of the manuscript. All authors have read and approved the final version of the manuscript.

II.2.7 ACKNOWLEDGEMENTS AND FUNDING

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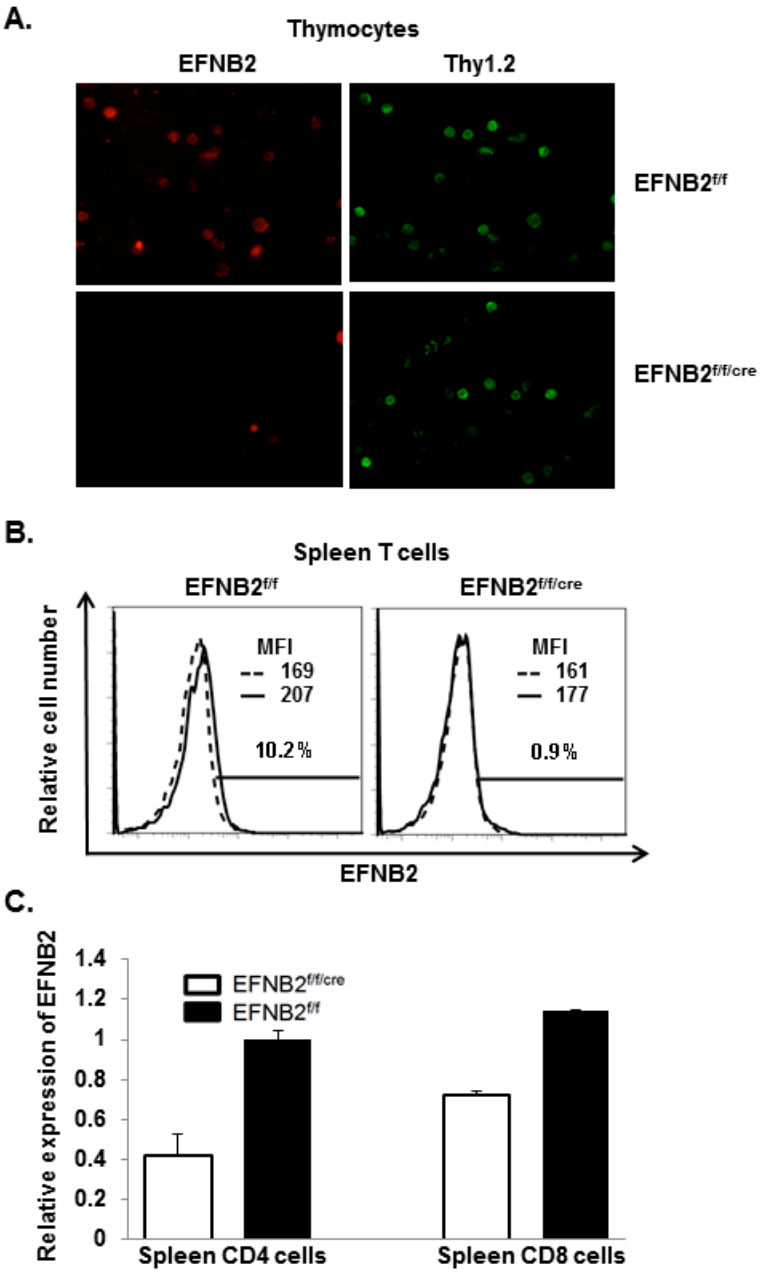
II.2.9 FIGURES and LEGENDS

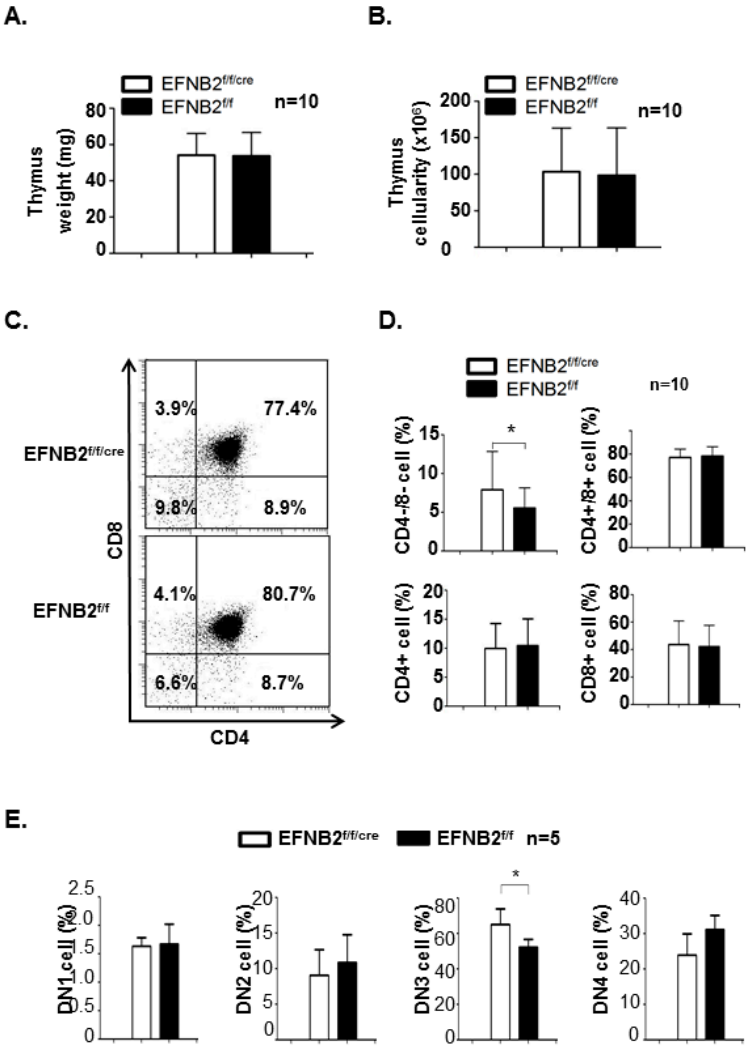
Figure 2.6. T cell-specific deletion of EFNB2 according to immunofluorescent microscopy, flow thytometry and RT/qPCR.



A. Thymocytes from WT (upper row) or KO (bottom row) mice were stained with FITC-rat anti-Thy1.2 (green) and PE-rat anti-EFNB1 (red) mAbs. Micrographs of the cells with pseudocoloring are shown. *B.* CD3-gated spleen T cells from *EFNB2$^{f/f/cre}$* mice or *EFNB2$^{f/f}$*

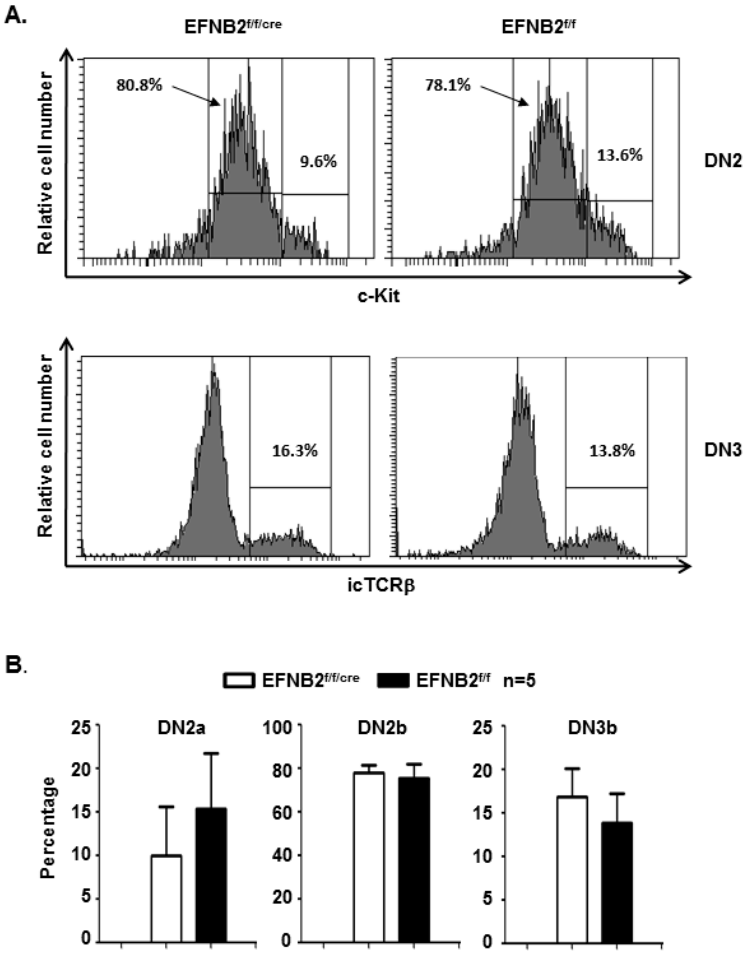
controls were analyzed for EFNB2 expression by flow cytometry. The dotted line represents isotypic control Ab, and solid line, anti-EFNB2 Abs. Mean fluorescence intensity and percentage of EFNB2 positive cells were indicated. The experiments were repeated three times and data from representative ones are shown. *C* Total RNA was extracted from spleen CD4 and CD8 T cells of *EFNB2*$^{f/f/cre}$ mice or *EFNB2*$^{f/f}$ controls. Their EFNb2 mRNA was analyzed by RT/qPCR using β-actin as an internal control. Data are expressed as the means + SD of the ratios of EFNb2/β- signals.

Figure 2.7. Phenotype of EFNB2 KO thymuses



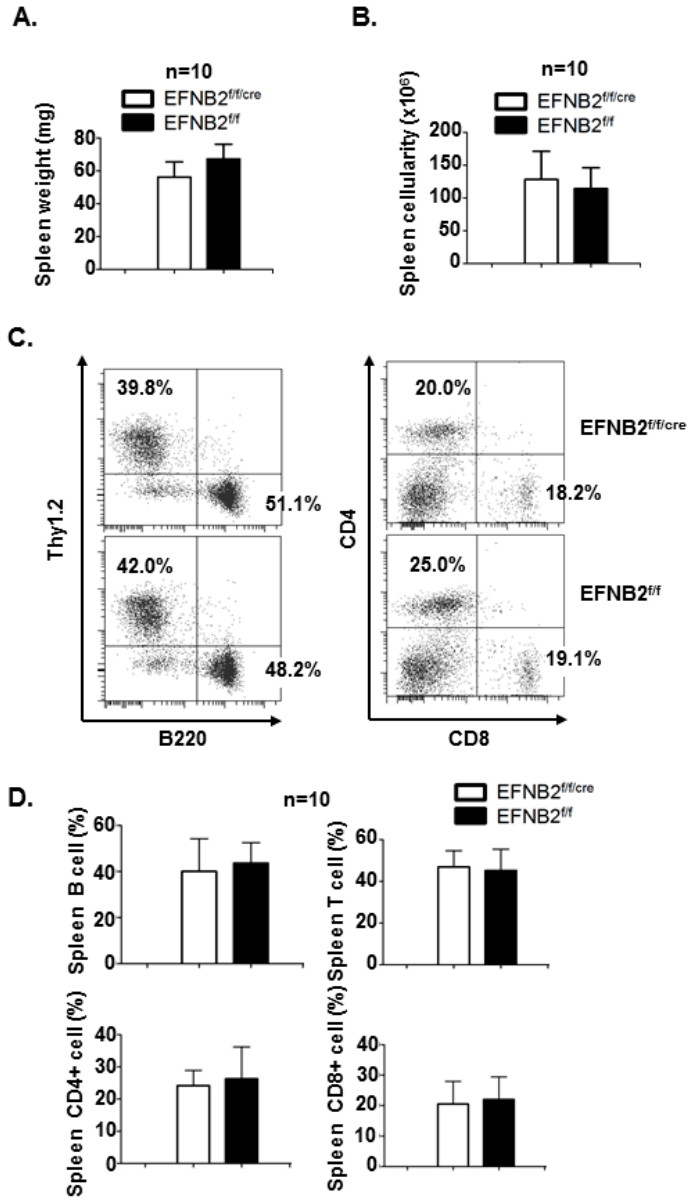
A and B. Thymus weight and cellularity of EFNB2 KO mice Thymus weight and cellularity of 10 pairs of EFNB2 KO and WT mice are presented. No statistically significant difference was found between the KO and WT mice (p>0.05, paired Student's t test). C and D. Subpopulations of thymocytes from EFNB2 KO and WT mice according to flow cytometry. Representative histograms of DN, DP, CD4 SP and CD8 SP cell populations are shown in C and bar graphs summarizing data from 10 pairs of KO and WT mice are illustrated in D (* p<0.05; paired Student's t test). E**.** The percentages of DN1, DN2, DN3 and DN4 cells in Lck-EFNB2$^{f/f}$ and EFNB2$^{f/f}$ mice from 5 independent experiments are shown in bar graphs (* p<0.05; paired Student's t test).

Figure 2.8. DN2 and DN3 subpopulations



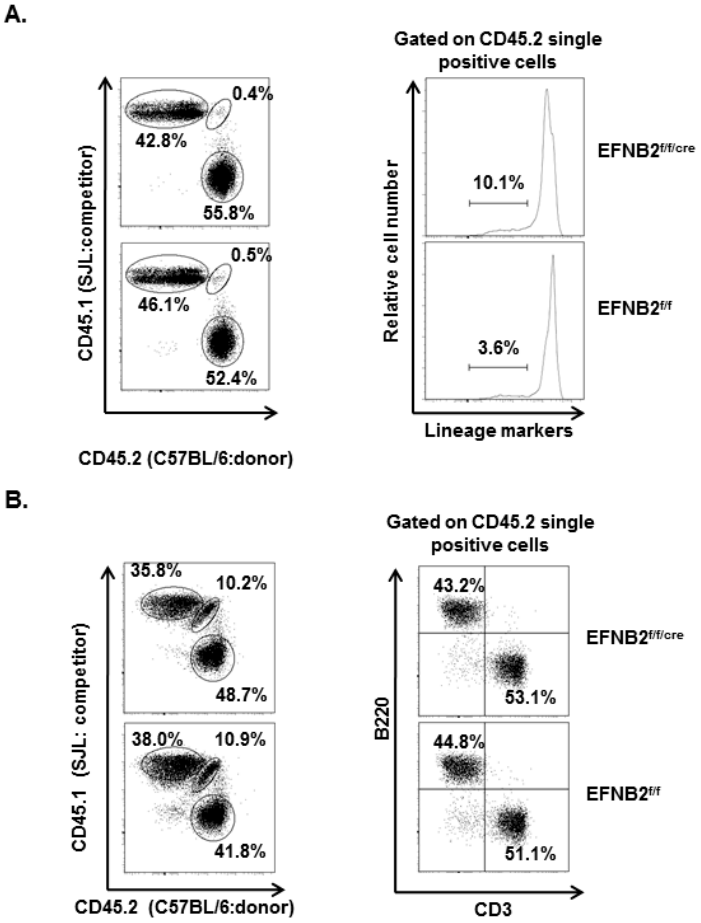
A. Flow cytometric analysis of c-Kit expression in DN2 subset and icTCRβ expression in DN3 subset. Data are representative of five independent staining. B. Data shown are the average±SD of five independent experiments. No statistically significant difference was obtained from KO and WT mice.

Figure 2.9. Phenotype of EFNB2 KO spleens



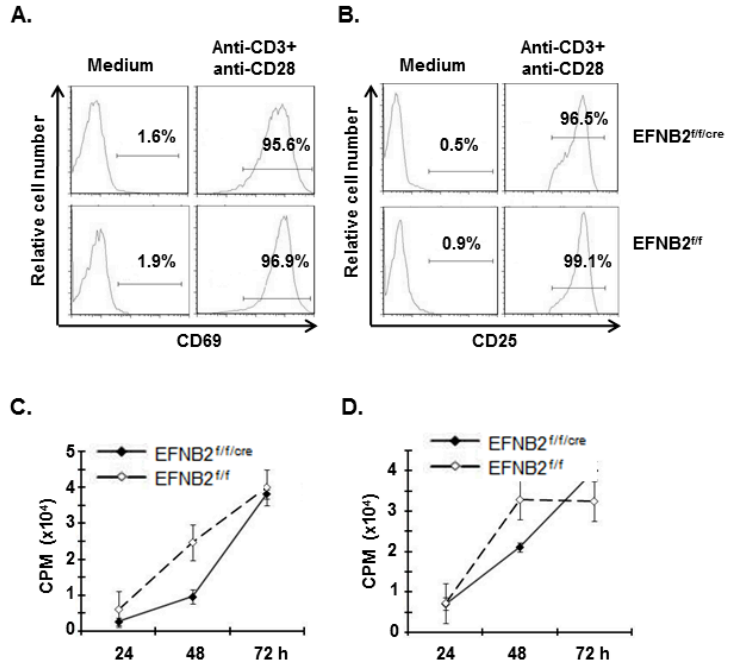
A and B. Spleen size and cellularity of EFNB2 KO and WT mice Spleen weight (A) and cellularity (B**)** of 10 pairs of EFNB2 KO and WT mice are shown ($p>0.05$ according to paired Student's t test). C-E. Spleen T cell, B cell, CD4 T cell and CD8 T cell populations of EFNB2 KO and WT mice according to flow cytometry. Populations of spleen $Thy1.2^+$ T cells and $B220^+$ B cells (C), and $CD4^+$ T cells and $CD8^+$ T cells (D) were analyzed by flow cytometry. Representative histograms are shown in C and D. Bar graphs summarizing data from 10 pairs of EFNB2 KO and WT mice are illustrated in E ($p>0.05$; paired Student's *t* test).

Figure 2.10. EFNB2 KO progenitors reconstitute the thymus and spleen in mixed chimeras



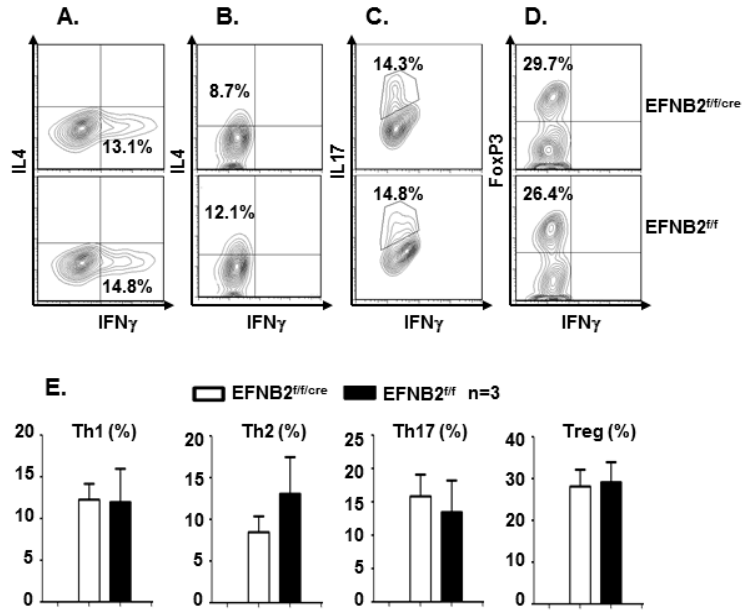
$1x10^6$ T-cell depleted EFNB2$^{f/f/cre}$ KO and EFNB2$^{f/f}$ WT bone marrow cells (CD45.2$^+$) were mixed with T-cell depleted bone marrow cells from B6.SJL competitor at 1:1 ratio, and transplanted to lethally irradiated C57BL/6 x SJL F1 recipients (n=3 for the first experiment; n=2 for the repeating experiments). After 8 to 10 weeks, cells from spleen were analyzed for CD45.2 and CD45.1 staining. Percentages of CD45.2$^+$ cells (derived from Lck-Cre-EFNB2$^{f/f}$ or EFNB2$^{f/f}$ bone marrow cells), CD45.1$^+$ cells (derived from competing B6.SJL bone marrow cells), and CD45.1$^+$/CD45.2$^+$ cells (derived from residue cells of the recipients) in thymocytes (A, left panal) and spleen cells (B, left panel) are indicated. Among CD45.2$^+$ cells, the percentage of lineage negative thymocytes was shown (A, right panel. B220$^+$ B cells and CD3$^+$ T cells among CD45.2$^+$ cells (derived from Lck-Cre-EFNB2$^{f/f}$ or EFNB2$^{f/f}$ bone marrow cells) in the spleen were determined by flow cytometry, and their percentage is shown (B, right panel).

Figure 2.11. Normal activation and proliferation of EFNB2 KO T cells



A and B. Activation marker expression in EFNB2 KO T cells Total spleen cells from EFNB2 KO and WT mice were stimulated with soluble anti-CD3 plus anti-CD28 mAbs for 16 hours. Thy1.2^{+} T cells were gated and analyzed for their CD69 (A) and CD25 (B) expression with 2-color flow cytometry. The percentages of CD69- and CD25-positive cells are indicated in the histograms.C and D. Normal proliferation of EFNB2 KO T cells Purified spleen T cells from EFNB2 KO or WT mice were cultured in wells coated with solid phase anti-CD3 mAb (C) or anti-CD3 plus anti-CD28 mAbs (D) for 24, 48 and 72 h. The cells were pulsed with ^{3}H-thymidine for the last 16 h of the culture, and ^{3}H-thymidine uptake was measured. Samples were in triplicate. Mean ± SD of cpm are shown. Experiments in this figure were repeated more than 5 times and representative data are shown.

Figure 2.12. Normal differentiation of EFNB2 KO CD4 cell in vitro



Naïve CD4 cells were cultured under conditions favoring Th1 (A), Th2 (B), Th17 (C) and Treg (D) cells. Their intracellular cytokine or FoxP3 expression was determined by flow cytometry on day 3 for Th1, Th17 and Treg, and day 5 for Th2. Representative data are shown in A. Experiments were repeated 3 times and bar graph (B) indicates the percentage ± SD ($p>0.05$).

II.3 Article 3

Effect of reduced EPHB4 expression in thymic epithelial cells on thymocyte development and peripheral T cell function

Wei Jin*, Hongyu Luo* and Jiangping Wu*+

Mol Immunol. 2013 Nov 15;58(1):1-9

Summary: The Eph kinase (EPH) and ephrin (EFN) families are involved in a broad range of developmental processes. Increasing evidence is demonstrating the important roles of EPHBs and EphrinBs in the immune system. In this study on epithelial cell-specific *Ephb4* knockout (KO) mice, we investigated T-cell development and function after EPHB4 deletion. KO mice presented normal thymic weight and cellularity. Their thymocyte subpopulation percentages were in the normal range. KO mice had normal T-cell numbers and percentages in the spleen, and T cells were activated and proliferated normally upon TCR ligation. Furthermore, naïve spleen CD4 cells from KO and wild type mice were capable of differentiating, in a comparable manner, into Th1, Th17 and Treg cells. *In vivo*, KO mice mounted effective delayed type hypersensitivity responses, indicating that thymocytes develop normally in the absence of TEC EPHB4, and T cells derived from EPHB4-deleted thymic epithelian cells (TEC) have normal function. Our data suggest that heavy redundancy and promiscuous interaction between EPHs and EFNs compensate for the missing EPHB4 in TECs, and TEC EPHB4's role in T cell development might only be revealed if multiple EPHs are ablated simultaneously. We cannot exclude the possibility that 1) some immunological parameters not examined in this study are affected by the deletion; 2) the deletion is not complete due to the leaky Cre-LoxP system, and the remaining EPHB4 in TEC is sufficient for thymocyte

development; or 3) EPHB4 expression in TEC is not required for T cell development and function.

WJ, HL, and JW participated in experimental procedures leading to figures contained within. WJ conducted all the experiments. WJ and JW participated in writing of the manuscript. All authors have read and approved the final version of the manuscript.

Effect of reduced EPHB4 expression in thymic epithelial cells on thymocyte development and peripheral T cell function

Wei Jin*, Hongyu Luo* and Jiangping Wu*+

Running title: EPHB4 gene knockout in thymic epithelial cells affects T cells minimally

ABSTRACT

The Eph kinase (EPH) and ephrin (EFN) families are involved in a broad range of developmental processes. Increasing evidence is demonstrating the important roles of EPHBs and EphrinBs in the immune system. In this study on epithelial cell-specific *Ephb4* knockout (KO) mice, we investigated T-cell development and function after EPHB4 deletion. KO mice presented normal thymic weight and cellularity. Their thymocyte subpopulation percentages were in the normal range. KO mice had normal T-cell numbers and percentages in the spleen, and T cells were activated and proliferated normally upon TCR ligation. Furthermore, naïve spleen CD4 cells from KO and wild type mice were capable of differentiating, in a comparable manner, into Th1, Th17 and Treg cells. *In vivo*, KO mice mounted effective delayed type hypersensitivity responses, indicating that thymocytes develop normally in the absence of TEC EPHB4, and T cells derived from EPHB4-deleted thymic epithelian cells (TEC) have normal function. Our data suggest that heavy redundancy and promiscuous interaction between EPHs and EFNs compensate for the missing EPHB4 in TECs, and TEC EPHB4's role in T cell development might only be revealed if multiple EPHs are ablated simultaneously. We cannot exclude the possibility that 1) some immunological parameters not examined in this study are affected by the deletion; 2) the deletion is not complete due to the leaky Cre-LoxP system, and the remaining EPHB4 in TEC is sufficient for thymocyte development; or 3) EPHB4 expression in TEC is not required for T cell development and function.

II.3.1 INTRODUCTION

Erythropoietin-producing hepatocyte kinases (EPHs) and their ligands ephrins (EFNs) are cell surface molecules. There are 15 EPHs and they are divided into A and B subfamilies according to sequence homology[11, 42, 88]. There are 9 EFNs, which are also cell surface molecules and are divided into A and B subfamilies. Members of the A subfamily attach to the cell surface through glycosylphosphatidylinositol anchoring, whereas members of the B subfamily attach through transmembrane tails [1-3]. EPHs and EFNs interact with each other by direct cell-cell contact, are capable of inducing bi-directional signalling between interacting cells, and are widely involved in various processes, including embryonic development[258], neuron axon and dendrite positioning[41, 259], wound repair[260], angiogenesis[71], bone metabolism[3, 11], intestinal epithelium maturation[261, 262], insulin secretion[263] as well as tumour metastasis[1-3]. Accumulating evidence from our laboratory and others has revealed Eph and EFN involvement also in the immune system [39, 40, 88, 89, 91-96, 264, 265].

EPHB4 was originally identified in human bone marrow $CD34^+$ cells and later in organs, such as the lungs, liver, kidneys, intestine, muscle, heart, thymus and brain[68, 266, 267]. Loss of EPHB4 and its preferred ligand EFNB2 results in early embryonic lethality with disturbed arterio-venous differentiation [268]. During angiogenesis, EPHB4 and EFNB2 are expressed on endothelial cells of veins and arteries, respectively[68]. EPHB4 has also been implicated in oncogenesis and tumour progression attributed to its angiogenesis-promoting effects[25], increasing survival [269], facilitating invasion and migration and/or regulating estrogen receptors [270].

A few studies have examined the role of EPHB4 in T-cell biology, but most of them provide indirect evidence. Our group demonstrated previously that solid-phase EFNB1, EFNB2 and EFNB3, ligands of EPHB4, can co-stimulate murine T cells[91-93]. Kawano et al. reported that in mice with EPHB1, B2, B3, and B6 deletion, solid-phase EFNB1 and EFNB2 promoted or inhibited anti-CD3-stimulated murine T-cell proliferation, depending on EFNB1 and EFNB2 concentration[99]. This implies that the remaining EPHB on the T-cell surface, i.e., EPHB4,

mediates such modulating effects of T cell proliferation. Human T cells seem to express EPHB4 at high levels, and EFNs on mesenchymal stem cell (MSC) surfaces, triggering EFNB2-mediated EPHB4 forward signaling, can repress T-cell activation by allogeneic antigens, according to indirect evidence of EFNB2 small interference RNA knockdown in MSCs and the use of pharmacological inhibitors presumably suppressing EPHB4 signaling[271].

Our investigation into the roles of various EPHs and EFNs in the immune system disclosed that EPHB4 was expressed at a low level in mouse thymocytes, but at a high level in thymic stroma cells. It raised an interesting question: is thymic epithelial cell (TEC) EPHB4 expression essential in thymocyte development and, consequently, mature T-cell function in the periphery? We generated conditional gene knockout (KO) mice with EPHB4 deletion in thymic epithelial cells (TECs). However, no significant anomaly was found in these animals in terms of thymocyte development and mature T-cell functions.

II.3.2 MATERIALS AND METHODS

Reverse transcription/quantitative polymerase chain reaction (RT-qPCR)

mRNA levels of *Efnb1*，*Efnb2, Efnb3, Ephb4* as well as *Ephb6* were measured by RT/qPCR. Total RNA from cells was extracted using TRIzol® (Invitrogen, Carlsbad, CA) and then reverse-transcribed with iScript™ cDNA synthesis kit (Bio-rad). Primers used are as follows:

Efnb1: 5'-TGCAACAAGCCACACCAGGAAATC- 3' (forward primer)
5'-CAAGCTCCCATTGGACGTTGATGT-3' (reverse primer)

Efnb2: 5'-CCCTTTGTGAAGCCAAATCCAGGT-3' (forward primer)
5'-TCCTGATGCGATCCCTGCGAATAA-3' (reverse primer)

Efnb3: 5'-AGTTCCGATCCCACCACGATTACT-3' (forward primer)
5'-TCCATGGGCATTTCAGACACAGGT-3' (reverse primer)

Ephb4: 5'-TTCCCTCGCCACTGCTTTAGAAGA-3' (forward primer)
5'-TGCCTGAGGGTAAGTCACCCATTT-3' (reverse primer)

Ephb6: 5'-AAGCCATAGCAGTGCCTCAGAACA-3' (forward primer)
5'-TCCAGAGCTAGAACTGATGACCCT-3' (reverse primer)

qPCR condition for the reaction was as follows: two minutes at 50°C, two minutes at 95°C followed by 15-30 cycles of 10 seconds at 94°C, 20 seconds at 58°C and 20 seconds at 72°C. Samples were tested in duplicate. β-actin mRNA level was used as internal controls and data were first calculated as signal ratios of tested gene mRNA/β-actin gene mRNA. The signal ratios of *Ephb6* versus *β-actin* of each experiment were normalized as 1, and expression levels of *Efnb* mRNA relative to *Ephb6* mRNA are presented.

Generation of epithelian-specific Ephb4 gene knockout mice

Details of *Ephb4* floxed mice generation and verification are reported elsewhere (manuscript in preparation). Mice with LoxP sites flanked by the 1st *Ephb4* exon were named *Ephb4$^{f/f}$*. They were backcrossed with C57BL/6 mice for 8 generations and then mated with keratin 5 (K5)-promoter-driven Cre transgenic (Tg) mice (strain Tg(KRT5-cre)1Tak) in the C57BL/6 background [272] to obtain epithelial cell-specific *Ephb4* gene KO mice, called *Ephb4* KO mice or simply KO mice in this study. *Ephb4$^{f/f}$* mice served as wild type (WT) controls.

All animals were housed under specific pathogen-free conditions and studied in accordance with a protocol (N09055JWs) approved by the Institutional Animal Protection Committee of the CRCHUM.

Flow cytometry

Single cell suspensions from thymi and spleens were prepared and stained for flow cytometry, as described in our previous publications[89, 93]. The cells were first stained with Abs against cell surface antigens fixed with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA), then with Abs against intracellular antigens. The Abs used for flow cytometry were: PE conjugated rat anti-mouse Thy1.2 mAB (0.2 μg/μl, Clone 53-2.1), APC-Cy™ 7-conjugated rat anti-mouse B220 mAb (0.2 μg/μl, Clone RA3-6B2), APC-conjugated hamster anti-mouse CD3 mAb (0.2 μg/μl, Clone 2C11), PerCP-conjugated rat anti-mouse CD4 mAb (0.2 μg/μl, Clone RM4-5), APC-conjugated rat anti-mouse CD8 mAB (0.2 μg/μl, Clone 53-6.7); APC-conjugated rat anti-mouse CD25 mAb (0.2 μg/μl, Clone 7D4), FITC-conjugated hamster anti-mouse CD69 mAb (0.2 μg/μl, Clone H1.2F3), FITC-conjugated mouse anti-human IFN-γ

mAb (0.2 μg/μl, Clone B27), and PE-conjugate rat anti-mouse IL-17 mAb (0.2 μg/μl, Clone TC11-18H10) from BD Bioscience Pharmingen (San Diego, CA, USA); Pacific blue-conjugated rat anti-mouse CD44 mAb (Clone 1M7) from BioLegend (San Diego, CA, USA); and APC-conjugated rat anti-mouse FoxP3 mAb (0.2 μg/μl, Clone FJK-16s) from eBioscience (San Diego, CA, USA).

T-cell activation and proliferation assay

Total spleen cells were stimulated with soluble hamster anti-mouse CD3 mAb (0.1μg/ml, Clone 2C11) plus rat anti-mouse CD28 mAb (1 μg/ml, Clone 37.51.1). After 16-h culture, the cells were analysed by flow cytometry for CD25 and CD69 expression. For T-cell proliferation, spleen T cells were purified with Easysep® Mouse T cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) and cultured in wells coated with hamster anti-mouse CD3 mAb (0.2 μg/ml for coating) plus rat anti-mouse CD28 mAb (1 μg/ml for coating). ^{3}H-thymidine uptake of cultured cells after 16-h ^{3}H-thymidine-pulse was measured at 24 h, 48 h and 72 h after culture, as described in our previous publication[93].

In vitro Th1, Th17 and Treg polarization

Th1, Th17 and Treg populations were polarized from naïve $CD4^{+}$ T cells isolated from splenocytes with Naïve $CD4^{+}$ T Cell Isolation Kits (R&D Systems). The purity of naïve $CD4^{+}$ cells was routinely greater than 95%. Purified naïve T cells (0.1×10^{6}/well) were mixed with T-cell-depleted, irradiated (3,000 Rads) C57BL/6 feeder splenocytes (0.5×10^{6} cells/well), and cultured in 96-well plates in RPMI medium 1640 containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin G, 1× non-essential amino acids, 1 μM sodium pyruvate, 2.5 μM β-mercaptoethanol, and 2 μg/ml soluble hamster anti-CD3ε mAb (Clone 2C11). Recombinant mouse IL-12 (10 ng/ml) and rat anti-mouse IL-4 mAb (10 μg/ml, Clone 11B11) were added to culture for Th1 polarization. Cultures were supplemented with recombinant mouse IL-6 (20 ng/ml), recombinant human TGF-β1 (5 ng/ml), rat anti-mouse IL-4 mAb (10 μg/ml) and rat-anti-mouse IFN-γ mAb (10 μg/ml) for Th17 polarization. Recombinant human TGF-β1 (5 ng/ml), rat anti-mouse IL-4 mAb (10 μg/ml) and rat anti-mouse IFN-γ mAb (10 μg/ml) were added to culture for Treg polarization. Recombinant cytokines and mAbs against

cytokines were all from R&D Systems. Five days after culture, 5 nM of PMA, 500 ng/ml of ionomycin, and protein transport inhibitor BD GolgiStop™ (BD Bioscience Pharmingen) were added for the last 4 h of culture, harvested and stained for CD4 and intracellular cytokines or FoxP3, for flow cytometry analysis.

Delayed type hypersensitivity (DTH) assay

DTH assay was undertaken according to our earlier publications[96]. Briefly, WT and KO littermates were sensitized by painting their shaved abdomens with 0.4 ml 0.5% FITC in acetone/dibutyl phthalate (1:1 ratio). After 6 days, DTH was elicited by painting both sides of an ear with 0.5% FITC. After 24 h, increased ear thickness was measured with a digital calliper (Mitutoyo Corporation, Kawasaki, Japan).

II.3.3 RESULTS

Ephb4 mRNA expression in thymocytes and thymic stroma

We assessed the expression of *Ephb4* and its ligands *Efnb1, Efnb2, Efnb3* at the mRNA level in thymocytes and thymic stroma by RT-qPCR. *Ephb6*, which is expressed at high levels in the thymus[97], was included as a reference for relative *Efnb4* expression levels. As illustrated in Figure 1, *Ephb4* mRNA expression was low in murine thymocytes, but was detected at a moderate level in thymic stroma, composed mainly of epithelial cells and connective tissues.

Ephb4 deletion in thymic medulla

Moderate-level *Ephb4* mRNA expression in thymic stroma gave rise to the question: is such expression essential for thymocyte development? To address it, we generated epiothelial cell-specific *Ephb4* KO mice with K5 (keratin 5)-promotor-driven Cre expression in mice with LoxP sites flanking the first exon of *Ephb4* gene. Although keratin 5 is predominately expressed in medullar but not cortex TEC in the thymus, this promoter drives the Cre expression in both medullar and cortex TEC [43], hence, in theory it should result in EPHB4 deletion in both thymic medullar and cortex TEC. *Ephb4* mRNA level reduction in the thymic stroma but not in the brain and kidney of the KO mice was evidenced in Figure 2A. The *Ephb4* mRNA deletion in the stroma was not complete, probably due to contaminating none

epithelial cells or insufficient strength of the promoter. These data illustrate TEC-specific reduction of EPHB4 expression in the KO thymus.

Cellularity and cell subpopulations in the thymus and spleen of EphB4 KO mice

Ephb4 KO mice were fertile and presented no obvious anomaly on visual inspection. The thymi of these mice were of similar weight and cellularity compared to those of WT controls (Fig. 3A). We analyzed thymocyte subpopulations by flow cytometry. Representative histograms appear in Figure 3B, and summarized data on 10 pairs of WT and KO mice appear in bar graphs in Figure 3C. No significant differences between WT and KO thymi were observed in regard to their CD4CD8 double-negative, CD4CD8 double-positive, CD4 single-positive and CD8 single-positive thymocyte subpopulations.

Spleen weight and cellularity in WT and *Ephb4* KO mice were not significantly different (Figs. 4A and 4B). Different spleen cell populations were assessed by flow cytometry. Representative histograms are presented in Figures 4C-4E), and data on 10 pairs of WT and KO mice are summarized in bar graphs in Figure 4F. T-cell ($Thy1.2^+$) and B-cell ($B220^+$) populations in KO spleens were comparable to those of WT spleens (Fig. 4C). No apparent difference was apparent between WT and KO spleens relative to percentages of CD4 and CD8 T cells (Fig. 4D) or γδ T cells and αβ T cells (Fig. 4E).

The results of this section show that EPHB4 deletion in TECs does not apparently influence thymocyte development in terms of subpopulation percentages and numbers, with no apparent consequences on peripheral T cells, again in terms of subpopulation percentages and numbers.

In vitro T-cell activation and proliferation were not compromised by EPHB4 deletion in TECs

We next investigated whether peripheral T cells derived from KO thymi which lacked EPHB4 expression in TECs. Total spleen cells from WT and KO mice were stimulated with soluble anti-CD3 plus anti-CD28 mAb. After 16-h culture, $Thy1.2^+$ T cells were gated and analyzed for their activation marker CD69 and IL-2Rα CD25 expression. Histograms from

representative experiments appear in Figures 5A and 5B, and data from 3 independent experiments are summarized in bar graphs at the bottom of the histograms. Without TCR stimulation (cells cultured only in medium), less than 5% of T cells expressed CD69 and CD25 in WT and KO T cells. After activation by anti-CD3, more than 85% of KO T cells were CD69- and CD25-positive, similarly to WT T cells.

We then compared WT and KO T-cell proliferation upon TCR stimulation. Spleen T cells were enriched with magnetic beads, and purity was normally above 95%. Purified T cells were cultured in wells coated with anti-CD3 plus anti-CD28 mAb, and their proliferation was measured at 24, 48, and 72 h according to ^{3}H-thymidine incorporation. T cells from WT and KO mice proliferated at comparable rates, as reported in a representative experiment (Fig. 5C) and in a bar graph on the left summarizing data from 3 independent experiments.

In vitro T-cell differentiation into Th1, Th17 and Treg cells was not compromised by EPHB4 deletion in TECs

We also assessed the differentiation ability of naïve CD4 T cells from KO mice. Naïve spleen CD4 T cells were isolated and cultured under conditions favouring Th1, Th17 and Treg polarization. After 3 days, CD4+ cells were stained with mAbs against either intracellular cytokines characteristic of Th1 (INF-γ; Fig. 6A) and Th17 (IL-17; Fig. 6B), or transcription factor FoxP3 characteristic of Treg (Fig. 6C) and analyzed by 3-colour flow cytometry. Histograms from representative experiments are presented, and data from 3 independent experiments are summarized in bar graphs at the bottom of the histograms. We found that naïve CD4 cells from KO mice had similar abilities as those from WT mice to differentiate into Th1, Th17 and Treg effector cells.

KO mice presented no compromise in DTH

Finally, we investigated T-cell function in KO mice *in vivo* by DTH assay. Mice were primed with FITC on their shaved abdomen and challenged 1 week later by FITC ear painting. DTH was quantified according to the degree of ear swelling. Based on results from 10 pairs of WT and KO mice, KO mice presented no compromise in DTH as their ears swelled to a similar degree as those of WT controls (Fig. 7).

II.3.4 DISCUSSION

In the present study, we investigated the consequences of TEC EPHB4 deletion in thymocyte development and peripheral T-cell function, using K5 promoter-directed, conditional *Ephb4* deletion in mice. Although EPHB4 was expressed at a moderate level in TECs, its deletion did not affect thymus structure and thymocyte subpopulations. Peripheral T cells derived from such KO thymi were not compromised in terms of subpopulation percentages, activation marker up-regulation, proliferation, differentiation into Th1, Th17 and Treg cells, and DTH responses *in vivo*.

Our laboratory has been investigating the functions of various EPHBs and EFNBs in the immune system, and we have previously reported the roles of EPHB6, EFNB1, and EFNB2 in T cell development and function, employing gene KO mice as models [39, 40, 96, 264, 265]. EPHB4 is expressed at high levels in human T cells, and there is evidence that the forward signalling received by EPHB4 in human T cells transmits negative signalling, dampening TCR-triggered T-cell responses [271]. However, the evidence is not unequivocal as the inhibitors and stimulators used are less than specific. We intended to study EPHB4 involvement in T-cell immune responses in T-cell-specific *Ephb4* conditional KO mice to draw more precise conclusions. However, an initial survey of the expression spectrum of EFNBs and some EPHBs showed that *Ephb4* mRNA was minimally expressed in murine thymocytes and peripheral T cells. The discrepancy of EPHB4 expression in the T-cell compartment between humans and mice could be simply due to species differences. Consequently, in mice, EPHB4 is unlikely to participate directly in T-cell immune responses, and perhaps its roles orthologous to human EPHB4 are subsumed by other members of the EPH family. However, we found that TECs expressed a moderate level of *Ephb4* mRNA. We, therefore, shifted our attention to TEC EPHB4 in thymocyte development and T-cell function.

Maturation of thymocytes involves migration of precursor cells between different compartments of the thymus along thymic stromal cells undergoing differentiation[273]. TECs

are a major component of thymic stroma, and interaction between TECs and thymocytes is vital in positive and negative thymocyte selection[147-149]. TEC dysfunction could have disastrous consequences in thymocyte development. *Whn* gene mutation in epithelial cells leads to a nude phenotype in mice, accompanied by total ablation of thymocytes and, consequently, peripheral T cells in these animals[274]. It should be mentioned that the interaction between thymocytes and TECs is bi-directional, i.e., thymocyte function could affect TEC structure and function[154-156]. We demonstrated previously that T-cell-specific EFNB1 and EFNB2 deletion not only affects the T-cell compartment, but also elicits abnormal medullary TEC structure[40]. With such information in mind, we crossed K5 promoter-driven Cre recombinase Tg mice and Floxed *Ephb4* mice, evoking epithelium-specific EPHB4 deletion, including TEC EPHB4 removal, to assess TEC EPHB4 involvement in the T-cell compartment. K5 is highly expressed in proliferating, stratified squamous epithelial cells[275]. In the thymus, K5 is predominantly expressed in medullary and cortico-medullary junction TECs[154]. However, the K5 promoter in Tg mice could drive gene expression in TECs of both the cortex and medulla [276], due either to a lack of a restricting element in the promoter sequence or to the fact that $K5^+$ TECs in the cortex are derived from $K5^+$ precursors. Whatever the reason, the end result is that EPHB4 expression in TECs of the thymus is reduced, if not completely deleted, in our KO mice. A lack of complete deletion could be due to insufficient promoter strength

Despite significantly reduced TEC EPHB4 expression, we did not observe any anomaly in thymocyte and peripheral T-cell development in terms of absolute cell numbers and percentages of their subpopulation composition. Peripheral T cells derived from thymi lacking EPHB4 in KO mice proliferated and differentiated into Th and Treg cells in a comparable manner as those from WT mice. There are 3 possible explanations for a lack of observed phenotype after EPHB4 deletion in TECs. The first is redundancy of the EPHB system. Other members of the EPHB or even the EPHA subfamily expressed on TECs could substitute for EPHB4 in its absence, to interact with EFNs on thymocytes, particularly EFNB2, the major ligand of EPHB4. Also, EFNB2 on thymocytes could receive signals from EPHBs other than EPHB4 from fraternal, neighbouring thymocytes to compensate for the loss of EPHB4 signaling from TECs. Of course, such compensation will not be suitable if TEC EPHB4's

function is to provide directional guidance to thymocyte migration within thymi during their maturation. The second possible explanation is that the deletion of EPHB4 in TEC is not complete, as shown in Figure 2A, due to insufficient potency of the K5 promoter for Cre transgene expression, and the remaining EPHB4 in TEC is sufficient for thymocyte development. The third possible explanation is that EPHB4 on TECs is not essential in T-cell development. Although this last explanation cannot be totally excluded, we favour the first or second. Through evolution, most, if not all, existing molecules should have a reason to be there. A lack of phenotype after gene deletion does not necessarily mean a lack of importance of this gene. On the contrary, it probably signifies that the function of this gene in a given process is so vital that heavy redundancy exists to prevent any accidental mutation causing disruption of the process, in this case, thymocyte development. The true function of EPHB4 in TECs will probably only be revealed after multiple EPHBs are deleted simultaneously and more thoroughly, as in the case of EFNB1 and EFNB2, for which single gene deletion in the T-cell compartment shows no apparent phenotype[264, 265], but a serious phenotype is revealed only after both of them are null-mutated[39, 40].

II.3.5 ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes of Health Research (MOP57697 and MOP123389), the Heart and Stroke Foundation of Quebec, the Natural Sciences and Engineering Research Council of Canada (203906-2012), the Juvenile Diabetes Research Foundation (17-2013-440) and the Jean-Louis Levesque Foundation to J.W. and by a grant from the Canadian Institutes of Health Research to H.L. (MOP97829).

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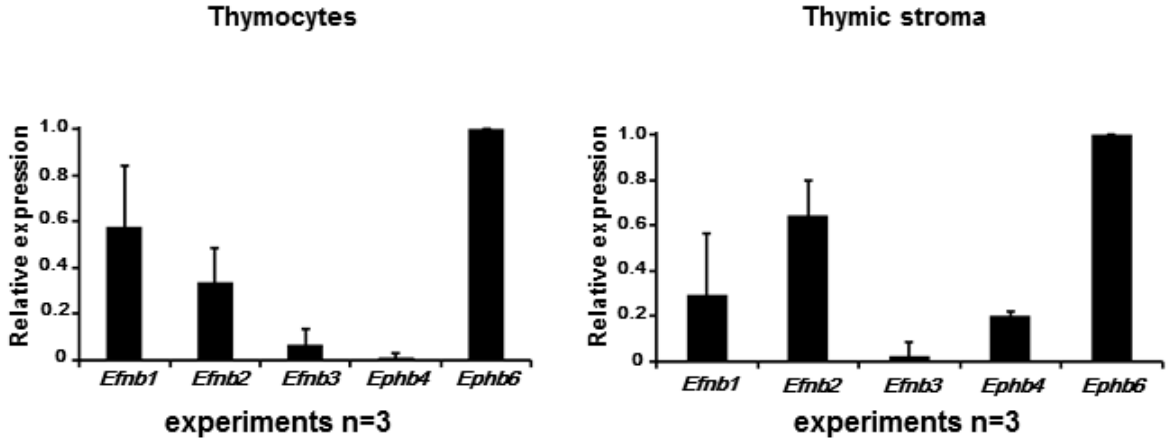
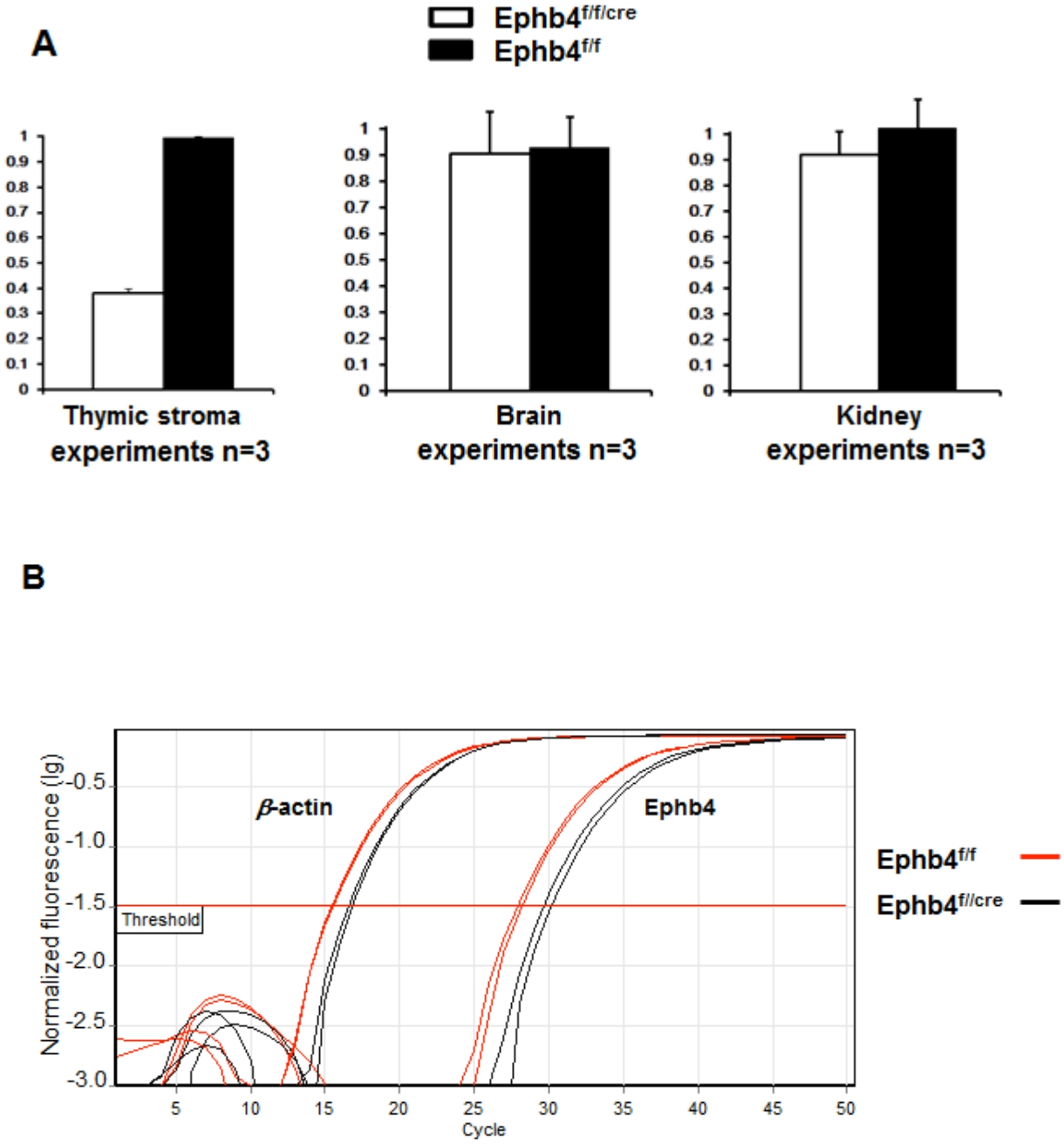
II.3.6 FIGURES and LEGENDS

Figure 2.13. mRNA expression of Ephb4 and related molecules in thymocytes and thymic stroma cells



Total RNA was extracted from thymocytes and thymic stroma (thymus after thymocytes were flushed out) of WT mice, and analyzed by RT-qPCR for the mRNA expression of *Ephb4, Ephb6, Efnb1, Efnb2* and *Efnb3*. The results are expressed as ratios of test gene versus β-actin signals with means ± SD from 3 pairs of KO and WT mice, followed by normalization of the ratios using the ratio of Ephb6/β-actin in each experiment as 1.

Figure 2.14. TEC-specific deletion of Ephb4 in KO mice according to RT-qPCR



A. Ephb4 mRNA levels in thymic stroma, brain and kidneys

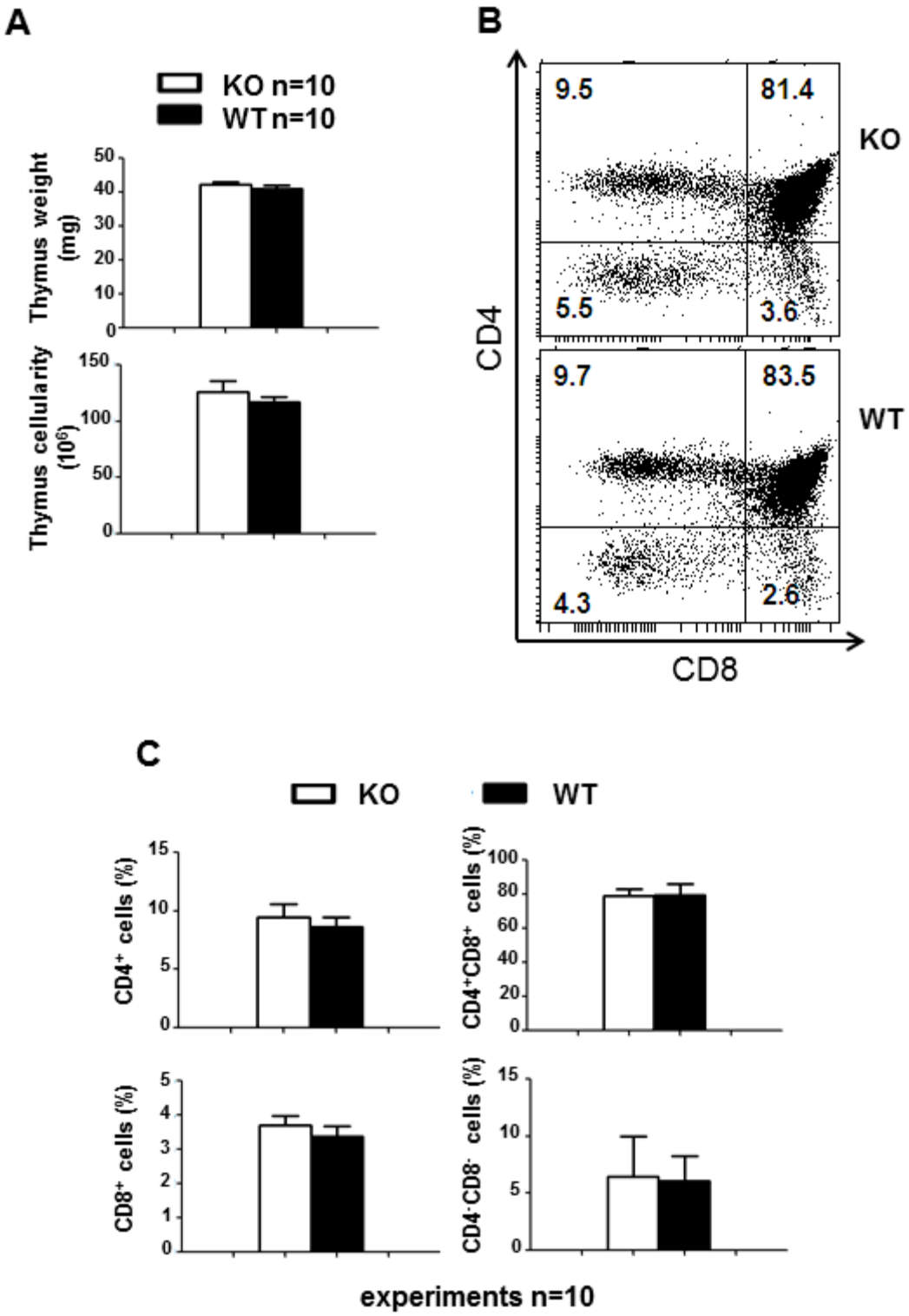
Total RNA was extracted from thymic stroma, brain and kidneys of WT and KO mice. The results are expressed as ratios of test gene versus β-actin signals with means ± SD from 3 pairs of KO and WT mice, followed by normalization of the ratios using the ratio of Ephb6/β-actin

in each experiment as 1. The experiments were performed twice and representative data are shown.

B. Real-time PCR curves of Ephb4 mRNA expression in WT and KO thymic stroma cells

The experiments were carried out as described in A. Typical real-time PCR curves showing the *Ephb4* and *β-actin* signals of the WT and KO stroma cells are presented.

Figure 2.15. Phenotype of EPHB4 KO thymi

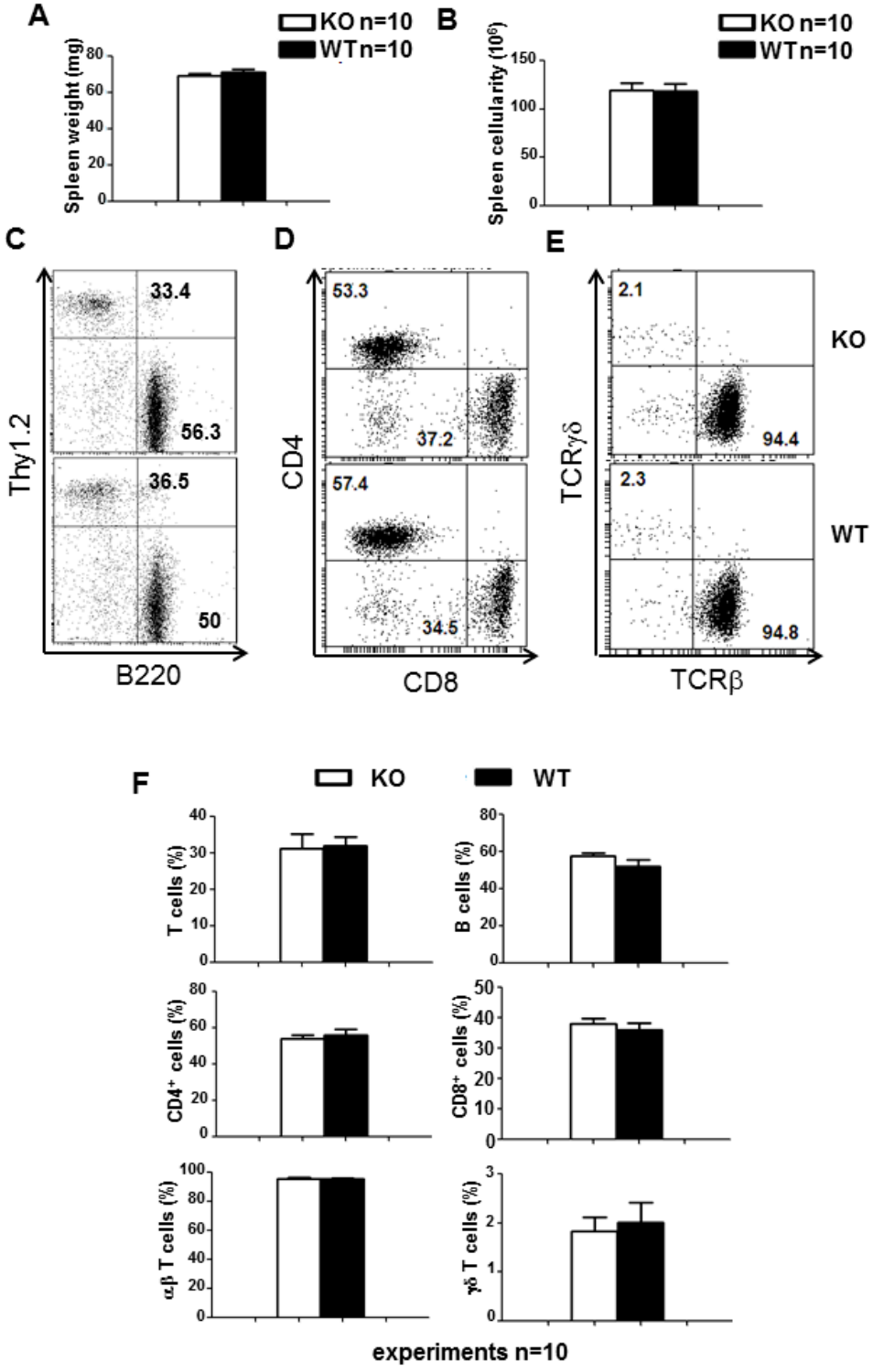


A. Thymus weight and cellularity of KO mice

N=10 pairs (p>0.05; paired Student's t test).

B and C. Subpopulations of thymocytes from WT and KO mice according to flow cytometry, Representative histograms are shown in B and bar graphs summarizing data from 10 independent experiments are illustrated in C (p>0.05; paired Student's t test).

Figure 2.16. Phenotype of EPHB4 KO spleens



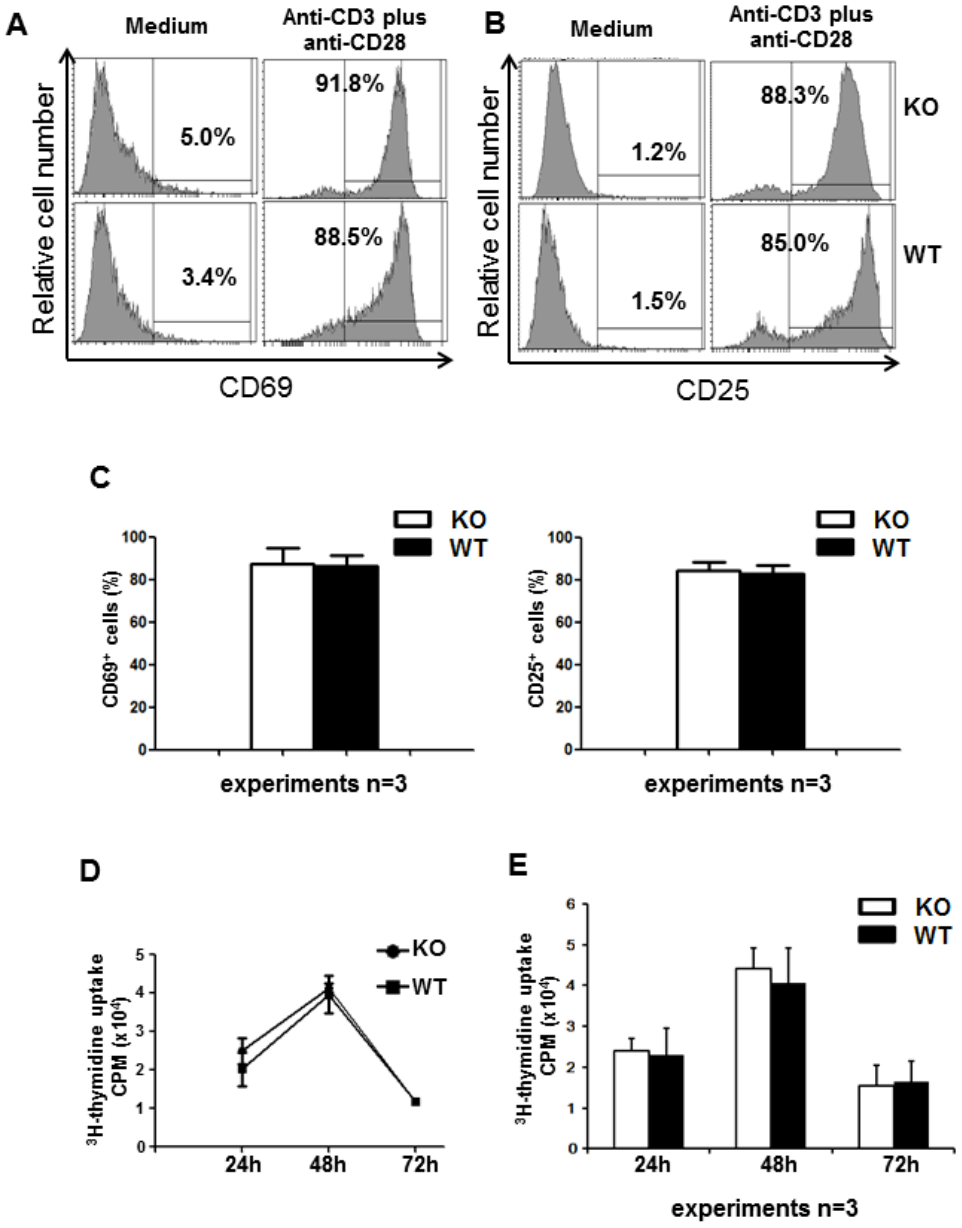
A and B. Spleen weight and cellularity of WT and KO mice

N=10 pairs ($p > 0.05$; paired Student's t test).

C, D and E. Spleen T-cell, B-cell, CD4 T-cell, CD8 T-cell, αβ T-cell and γδ T-cell populations from WT and KO mice according to flow cytometry.

Representative histograms are shown in C (Thy1.2$^+$ T cells versus B220$^+$ B cells). D: CD4 cells versus CD8 cells. E: αβ T cells versus γδ T cells. Bar graphs summarizing data from 10 independent experiments are illustrated in F (p>0.05; paired Student's t test).

Figure 2.17. Normal activation and proliferation of KO T cells



A-C. Activation marker expression

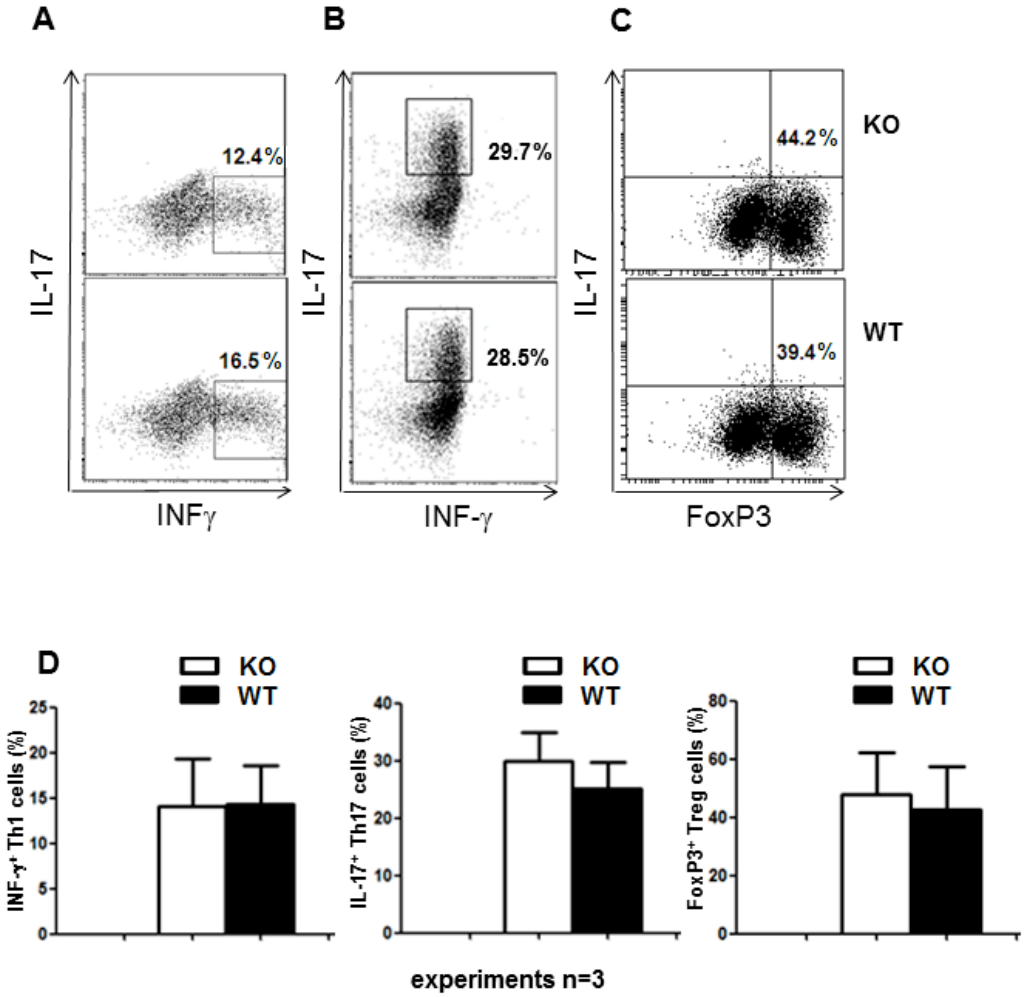
Total spleen cells from WT and KO mice were stimulated with soluble anti-CD3 and anti-CD28 mAbs for 16 h, and stained with PE-conjugated rat anti-mouse Thy1.2 mAb and FITC-conjugated rat anti-mouse CD25 or CD69 mAbs, followed by 2-colour flow cytometry. Representative histograms show CD69 (A) and CD25 (B) expression in Thy1.2-gated T cells.

Bar graphs summarizing data from 3 independent experiments are illustrated in C (p>0.05; paired Student's t test).

D and E. T-cell proliferation

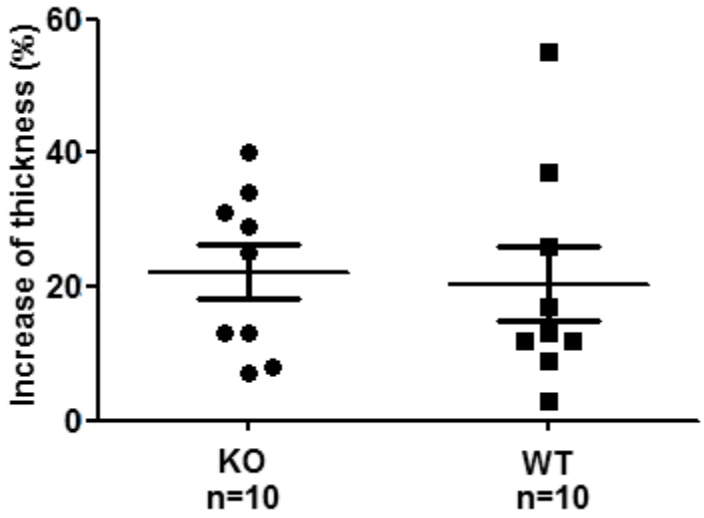
Purified spleen T cells from WT and KO mice were cultured in wells coated with anti-CD3 mAb (0.2 μg/ml for coating) and anti-CD28 mAb (1 μg/ml for coating) for 24 to 72 h. The cells were pulsed with ^{3}H-thymidine for the last 16 h of culture, and then harvested for ^{3}H-thymidine uptake assay. Samples were in triplicate. Means ± SD of cpm are shown. Experiments were repeated more than 3 times, and representative data are reported (D). Data from 3 independent experiments are summarized in a bar graph (E) (p>0.05; paired Student's t test).

Figure 2.18. Naïve KO T cells differentiated normally into Th1, Th17 and Treg cells



Naïve WT and KO CD4 cells were cultured under conditions favouring Th1, Th17 and Treg differentiation. After 5 days, 5 nM of PMA, 500 ng/ml of ionomycin, and the protein transport inhibitor BD GolgiStop™ were added for the last 4 h of culture, and the cells were stained for CD4 and intracellular IFN-γ, IL-17 and FoxP3, followed by 2-colour flow cytometry. Representative histograms of Th1 (A), Th17 (B) and Treg (C) cell differentiation are presented, and data from 3 independent experiments are summarized in a bar graph (D) (p>0.05; paired Student's t test).

Figure 2.19. KO mice mounted normal DTH responses



WT and KO mice were sensitized with FITC applied on abdomen skin. After 6 days, the ears were re-challenged with FITC, and ear thickness immediately before and 24 h after re-challenge was measured 3 times on each occasion. The percentage increase in ear thickness of each mouse was calculated as follows:

% increase = (mean ear thickness after FITC re-challenge – mean ear thickness before FITC re-challenge)/mean ear thickness before FITC re-challenge.

The dot graph summarizes data on 10 pairs of WT and KO mice. Mean $\pm$ SE of percentage ear thickness increase are indicated. No statistically significant difference is found between WT and KO groups ($p>0.05$; Student's *t* test).

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II.4 Article 4.

EFNb1 and EFNb2 are associated with IL-7Rα and retard its internalization from the cell surface

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Summary: In this study, the role of ephrins in regulating IL-7Rα expression was investigated. EFNb1 and EFNb2 physically interacted with IL-7Rα and regarded its IL-7-induced internalization. We found that EFNb1 and EFNb2 regulate IL-7R signaling by stabilizing IL-7Rα expression.

WJ generated lentiviral vectors which express different EFNB1 and EFNB2 truncations. Produced lentivirus was used to generate data which validate the observation of overexpression of EFNB1/B2 in EL4 cells.

EFNb1 and EFNb2 are associated with IL-7Rα and retard its internalization from the cell surface

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Running title: EFNb1 and EFNb2 bind to IL-7Rα

Background:

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

Results:

EFNb1 and EFNb2 physically interacted with IL-7Rα and regarded its IL-7-induced internalization.

Conclusion:

EFNb1 and EFNb2 regulate IL-7R signaling by stabilizing IL-7Rα expression.

Significance:

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

IL-7 plays vital roles in thymocyte development, T cell homeostasis and the survival of these cells. IL-7 receptor alpha (IL-7Rα) on thymocytes and T cells is rapidly internalized upon IL-7 ligation. Ephrins

(EFNs) are cell surface molecules and ligands of the largest receptor kinase family, Eph kinases. We discovered that T cell-specific double gene knockout of EFNb1 and EFNb2 (dKO) in mice led to reduced IL-7Rα expression in thymocytes and T cells, and that IL-7Rα down-regulation was accelerated in dKO CD4 cells upon IL-7 treatment. On the other hand, EFNb1 and EFNb2 over-expression on T cell lymphoma EL4 cells retarded IL-7Rα down-regulation. dKO T cells manifested compromised STAT5 activation and homeostatic proliferation, an IL-7-dependent process. Fluorescence resonance energy transfer and immunoprecipitation demonstrated that EFNb1 and EFNb2 interacted physically with IL-7Rα. Such interaction likely retarded IL-7Rα internalization, as EFNb1 and EFNb2 were not internalized. Therefore, we revealed a novel function of EFNb1 and EFNb2 in stabilizing IL-7Rα expression at the post-translational level, and a previously unknown *modus operandi* of EFNbs in the regulation of expression of other vital cell surface receptors.

II.4.1 INTRODUCTION

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

Ephs are the largest family of cell surface receptor tyrosine kinases, comprising about 25% of known receptor tyrosine kinases (9; http://cbweb.med.harvard.edu/eph-nomenclature/cell_letter.html). A total of 15 Ephs are classified into A and B subfamilies according to their sequence homology; the former has 9 members and the latter, 6, although not all are expressed in a given species (10,11). The ligands of Ephs, ephrins (EFNs), are also cell surface molecules (9). Nine EFNs are divided into A and B

subfamilies according to the way they anchor to the cell surface. The EFNA subfamily has 6 members that are glycosylphosphatidylinositol-anchored membrane proteins; the EFNB subfamily has 3 members that are transmembrane proteins.

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

Although they are ligands, EFNs can also transduce signals into cells (10,11) in a phenomenon known as "reverse signaling". Interaction between Eph and EFNs results in signaling in both directions, hence, bi-directional signaling. Since Ephs and EFNs are both cell surface molecules, they will normally be activated locally by their binding partners expressed on opposing cells during physical contact. Consequently, the major functions of Ephs and EFNs are related to pattern formation; however, additional functions unrelated to pattern formation have recently been observed.

Most reported functions of Ephs occur in the central nervous system where they are expressed in neurons and control axon and dendrite positioning (10,11). They are essential in the development of neuronal connections, circuit plasticity and repair. Some Ephs and EFNs also play important roles in other cells and organs, as described below and reviewed by Pasquale (13). They are expressed on endothelial cells and are vital in angiogenesis during normal embryonic development as well as in tumorigenesis. Intestinal epithelial cells express different levels of some Eph and EFNb family members that modulate the movement of epithelial cells along the crypt axis to maintain epithelium self-renewal. Ephb2 and EFNb2 are expressed on the endoderm during embryonic development and their bidirectional interaction is essential in urorectal development. Pancreatic β-cells communicate with each other via Epha and EFNa family members to synchronize their insulin secretion in response to blood glucose fluctuations. Several Ephb and EFNb family members are expressed on osteoclasts and osteoblasts where they regulate bone development, maintenance and repair. Multiple Eph and EFN members have been found to be expressed in some cancer cells and they appear to influence cancer cell growth. Ephb4 and EFNb2 are expressed on hematopoietic progenitor cells and regulate red blood cell production in response to hypoxia. EFNb1 and Epha4 expression in platelets contributes to the clotting process. EFNb1 expression on kidney epithelial cells (podocytes) likely plays a role in glomerular

filtration. Interaction between Ephb2 and EFNb2 regulates the ionic homeostasis of vestibular endolymph fluid in the inner ear.

Our group and others have reported that Ephs and EFNs, particularly their B family members, as well as some A family members are expressed in thymocytes and T cells; they are capable of modulating T-cell responses and survival (for a comprehensive review on the role of Eph/EFN in the immune system, see ref. 14). We have shown that EFNb1, EFNb2 and EFNb3 forward signaling through their Eph receptors can co-stimulate peripheral T cells by enhancing cytokine production and proliferation *in vitro*. We have also demonstrated that one of these EFN receptors, Ephb6, although lacking kinase activity, can transmit signals into T cells, and that its null mutation results in compromised T-cell responses *in vitro* and *in vivo*. However, Ephb6 null mutants have normal thymus structure and thymocyte development, probably due to complementary functions of other Eph family members.

In the present study, we discovered that EFNb1 and EFNb2 interact with IL-7Rα on the cell surface and such interaction delays internalization of the latter upon IL-7 stimulation. The significance of this finding is discussed.

II.4.2 MATERIALS AND METHODS

Flow cytometry

Single cell suspensions from the thymus, spleen or lymph nodes as well as T cell lymphoma EL4 and CHO cells were prepared and stained for flow cytometry as described in our previous publication (15). Goat anti-mouse EFNb1, goat anti-mouse EFNb2, and PE-donkey anti-goat IgG Abs were from R & D Systems (Minneapolis, MN). Biotinylated rat monoclonal antibodies (mAbs) in the mouse lineage panel kit as well as the following antibodies (Abs), FITC-mouse anti-mouse CD45.1 (clone A20), APC-rat anti-mouse CD25 (clone PC61), FITC-rat anti-mouse CD25 (clone 7D4), PE-rat anti-mouse CD4 (clones GK1.5 and H129.19), PerCP-rat anti-mouse CD4 (clone RM4-5), biotin-rat anti-mouse CD8b (clone 53-5.8), APC-Cy7- or FITC-rat anti-mouse CD45R/B220 (clone RA3-6B2), PE- or APC-hamster anti-mouse CD3ε (clone 145-2C11), PerCP-Cy5.5-mouse anti-mouse CD45.2 (clone 104), biotin- or FITC-rat anti-mouse CD44 (clone 1M7), FITC- or PE-rat anti-mouse CD8a (clone 53-6.7), and APC-rat anti-mouse CD8a (clone H57-597) were from BD Biosciences (San Diego, CA).

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

Generation of bone marrow chimeras

Eight- to 10-week-old C57BL/6 ($CD45.2^+$) x C57B6.SJL ($CD45.1^+$) F1 mice were irradiated at 1,100 Rads. Twenty-four h later, they received $4x10^6$ T cell-depleted bone marrow cells in a 1:1 ratio from C57/B6.SJL and dKO mice of the C57BL/6 background. WT mouse bone marrow was used as control. Eight to 10 weeks after bone marrow transplantation, spleen cells of the recipients were analyzed by flow cytometry.

Immunoblotting and immunoprecipitation

dKO or WT control spleen T cells and CD4 cells were purified with EasySep™ T Cell Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) or Miltanyi magnetic beads (Miltanyi Biotech, Bergisch Gladbach, Germany), respectively. These cells were reacted with or without IL-7 (20 ng/ml) at 37°C for the periods indicated. The cells were then lysed in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Complete™ Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail, Roche Diagnostics, Laval, QC, Canada). The lysates were resolved on 8% SDS-PAGE. Proteins on the gels were transferred to nitrocellulose membrane and blotted with rabbit anti-mouse Phospho-STAT5 (Tyr-694) mAb (clone C11C5; 1:1,000 dilution; Cell Signaling Technology, Danvers, MA) followed by horse radish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Baie-D'Urfe, QC, Canada). The membranes were then stripped and re-blotted with rabbit anti-mouse STAT5 mAb (clone 3H7; 1:1,000 dilution; Cell Signaling Technology) to assess total STAT5 expression. Signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

For immunoprecipitation, the cells were lysed and the lysates (500 μg per sample) were precipitated with rabbit anti-Myc polyclonal Ab-coated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA)

at 4°C for 4 h. The precipitated proteins were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes, which were blotted with HRP-conjugated rat anti-HA mAb (clone 3F10, Roche Diagnostics). The membranes were then stripped and re-blotted with HRP-conjugated rabbit anti-Myc Ab (Santa Cruz Biotechnology) for the detection of Myc-tagged proteins.

Assessment of in vivo T cell proliferation

In vivo T cell proliferation was monitored with CFSE labeling. T cells from dKO mice or from control WT mice in the C57BL/6 background ($CD45.2^+$) were labeled with CFSE (5 μM for 5 min at room temperature). Four million labeled T cells in 200 μl PBS were transferred IV to sub-lethally irradiated (600 Rads) B6.SJL mice ($CD45.1^+$). Six days later, recipient spleen cells were stained with Pacific Blue®-rat anti-CD45.2, PerCP-rat anti-CD4 or APC-rat anti-CD8 mAbs, and analyzed by 4-color flow cytometry.

IL-7Rα internalization in CD4 cells and EL4 cells

WT naïve CD4 cells from lymph nodes were first activated in wells pre-coated with hamster anti-mouse CD3ε mAb (clone 145-2C11, BD Biosciences) and rat anti-mouse CD28 mAb (clone 37.51.1, Cedarlane Laboratories) for 48 h to increase cell cytoplasm content for better visualization during imaging. The cells were washed and left resting for 4 days until their IL-7Rα expression returned to the pre-stimulation level. These activated CD4 cells and EL4 cells were cultured in medium with or without IL-7 (20 ng/ml) for 1-3 h, washed and stained with FITC-rat anti-mouse CD4 mAb (clones GK1.5, BD Biosciences) or FITC-rat anti-mouse Thy1.2 mAb (clone 53-2.1, BD Biosciences). The cells were then fixed with 4% paraformaldehyde on ice for 30 min and permeabilized for 30 min with ice-cold PBS containing 0.3% Triton-X100. After being blocked with 0.25% gelatin/0.01% saponin/1% rat serum (blocking buffer), they were incubated for 1 h with biotinylated rat anti-mouse IL-7Rα (clone SB/199, BioLegend), washed in blocking buffer and stained with streptavidin-conjugated Alexa Flour-568 for an additional h. Fluorescence signals were detected with a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA).

Quantitative reverse transcription/polymerase chain reaction (RT/qPCR)

IL-7Rα mRNA levels were measured by RT/qPCR. Forward primer 5' GGATGGAGACCTAGAAGATG 3' and reverse primer 5' GAGTTAGGCATTTCACTCGT 3' were

used to generate a 175-bp fragment for mouse IL-7Rα. PCR conditions for the reactions were as follows: 2 min at 50°C, 2 min at 95°C, followed by 45 cycles of 10 s at 94°C, 20 s at 58°C and 20 s at 72°C. β-actin mRNA levels were considered as internal controls. The data were first calculated as signal ratios of EFNb1 mRNA/β-actin mRNA and EFNb2 mRNA/β-actin mRNA. These ratios in different experiments were then normalized, and the final data were expressed as relative expression of IL-7Rα mRNA with the signal ratio of WT cells set as 1 unit.

EFNb1 and EFNb2 over-expression in CHO cells

PCR fragments containing the full-length coding sequences of EFNb1 and EFNb2 were retrieved from cDNA clones 3602195 and 6827408 from Open Biosystems (Huntsville, AL), employing the primer pairs 5'-ACGCG GCCGC ATCCT GAAGT-3'(forward)/5'-ACCTC GAGCA TGCTG GG-3'(reverse) and 5'- ACGCG GCCGC AGAAC TGGGA GCGGC TTGG-3'(forward)/5'-ACCTC GAGAA GAACA AGGTG -3' (reverse), respectively. Fragments were first subcloned into pCR-TA Vector® (Invitrogen, Burlington, ON, Canada) and subsequently into an episomal mammalian expression vector pCEP4™ (Invitrogen) at NotI and XhoI sites downstream of the CMV promoter. The resulting plasmids were named pCEP-EFNb1 and pCEP-EFNb2. EL4 cells were transfected with these constructs by electroporation. The transfectants were selected with hygromycin B to achieve stable EFNb1 and EFNb2 over-expression. For EFNb1 and EFNb2 over-expression in CHO cells, the full-length coding sequences of EFNb1 and EFNb2 were cloned into p-ReceiverM08 (Genecopoeia, Rockville, MD), and constructs named p-Receiver-EFNb1-Myc and p-Receiver-EFNb2-Myc served for stable expression of C-terminal Myc-tagged EFNb1 and EFNb2. The stable transfectants were selected with G418, and then transiently transfected with p-ReceiverIL-7Rα-HA (Genecopoeia) for C-terminal HA-tagged IL-7Rα expression; the cells were harvested after 30 h for immunoprecipitation studies.

Cell preparation and FRET

The direct association between IL-7Rα and EFNb1 or EFNb2 was assessed by FRET. EFNb1-EL4 and EFNb2-EL4 cells were first incubated on ice for 45 min with biotinylated rat anti-IL-7Rα mAb (clone SB/199, BioLegend) and goat anti-mouse EFNb1 Ab or goat anti-mouse EFNb2 Ab (both from R & D Systems), respectively. As negative controls, EFNb1-EL4 and EFNb2-EL4 cells were incubated with biotinylated rat anti-mouse Thy1.2 mAb (clone 30H12, BioLegend) and goat anti-mouse EFNb1 Ab or

goat anti-mouse EFNb2 Ab, respectively. The test and control cells were reacted with Alexa Flour-488-conjugated streptavidin for IL-7Rα (for both cross-linking and staining of IL-7Rα; Invitrogen), and with rhodamine-conjugated donkey anti-goat IgG $F(ab')_2$ fragments (for both cross-linking and staining of EFNb1 and EFNb2; Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C. The cells were transferred to a 37°C water bath and incubated for 10 min to allow cross-linking to occur; then, they were immediately fixed with 4% paraformaldehyde. Finally, the cells were washed, mounted on glass slides, and the FRET signal was examined under a Leica TCS SP5 laser-scanning confocal microscope. Alexa Flour-488 was the donor fluorophore, and rhodamine, the acceptor fluorophore.

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

AB FRET efficiency was calculated by fluorescence intensity of the donor before (D_{pre}) and after (D_{post}) acceptor-selective photo bleaching, according to the following formula: AB FRET efficiency = $(D_{post}-D_{pre})/D_{post}$

SE FRET efficiency was calculated based on the formula described by Woulters et al. (16):

SE FRET efficiency = (B-Axb-Cxc)/C

where B is FRET (indirect acceptor signal); A is donor emission (donor signal); b is the donor emission crosstalk ratio obtained from samples stained with donor fluorescence only: $b= B^{donor}/A^{donor}$; C is acceptor emission (direct acceptor signal); c is the acceptor excitation crosstalk ratio obtained from samples stained with acceptor fluorescence only: $c=B^{acceptor}/C^{acceptor}$.

II.4.3 RESULTS

Reduced expression of IL-7Rα in thymocytes and peripheral T cells from T cell-specific EFNb1 and EFNb2 double gene knockout (dKO) mice

To study the function of EFNb1 and EFNb2 in the T cell compartment, we generated conditional dKO mice with T cell-specific deletion of EFNb1 and EFNb1, using a proximal Lck promoter-driven Cre recombinase system. The proximal Lck promoter-caused EFN1 and EFNb2 deletion became effective starting from DN3 (17). Mice with floxed EFNb1/EFNb1 served as controls and were designated hereafter as wild type (WT). dKO mice manifested a significant phenotype, including about a 2-fold decrease in thymus weight and cellularity, and about a 2-fold reduction in spleen weight and spleen T cell number (18). Interestingly, we noticed that IL-7Rα expression was suppressed in most dKO thymocyte subpopulations and the decline was significant in DN3, DN4, CD4SP and CD8 SP cells (Fig. 1A). DP cells are known to have low IL-7Rα expression (19), which could explain the less than significant diminution of this subpopulation. In the periphery, $CD3^+$ T cells but not B cells from the control spleen expressed high IL-7Rα levels (Fig. 1B). IL-7Rα expression was significantly decreased in dKO spleen $CD3^+$ T cells.

Since moderate T lymphopenia (18) occurred in dKO mice, it might in theory trigger a compensatory increase of IL-7 secretion, which could, in turn, down-regulate IL-7Rα in T cells. To investigate this possibility, we co-transplanted dKO or WT bone marrow cells in the CD45.2 background (C57BL/6) along with bone marrow cells from B6.SJL mice of the CD45.1 background into lethally-irradiated C57BL/6 x SJL F1 recipients. Cells from dKO donors competed poorly with cells from B6.SJL donors to develop into mature T cells (Fig. 1C). Spleen CD4 and CD8 cells derived from WT donor cells had IL-7Rα expression similar to that derived from co-transplanted B6.SJL donor cells (Fig. 1C, upper panel), while CD4 and CD8 cells derived from dKO donor cells manifested reduced IL-7α expression, compared to cells derived from B6.SJL donor cells (Fig. 1C, lower panel). As competitor B6.SJL T cells and dKO T cells shared the same in vivo environment, the IL-7Rα down-regulation observed in dKO mice is not due to possibly increased IL-7 levels in vivo in these mice.

Does reduced IL-7Rα in dKO T cells compromise IL-7R signaling? As illustrated in Figure 1D, upon IL-7 stimulation, spleen T cells and CD4 cells from dKO mice indeed presented dampened STAT5 phosphorylation, a critical signaling event in the IL-7R signaling pathway.

We then assessed the functional consequence of reduced IL-7Rα expression in T cells in vivo. IL-7 is vital for T cell homeostatic expansion (2). We transferred carboxyfluorescein succinimidyl ester

(CFSE) labeled dKO or WT spleen T cells into sub-lethally irradiated B6.SJL mice. As seen in Figure 1E, the transferred dKO CD4 and CD8 T cells both showed significantly lower homeostatic proliferation *in vivo* compared to control WT T cells. Such compromised proliferation was consistent with the failed competition of dKO cells against B6.SJL cells as depicted in Figure 1C. This suggests that reduced IL-7Rα expression in T cells does have functional consequences and contributes to compromised homeostatic expansion in vivo.

EFNb1 and EFNb2 retard IL-7Rα internalization in T cells

The mechanism by which EFNb1 and EFNb2 modulate IL-7Rα expression was investigated herewith. Rapid internalization of IL-7Rα upon IL-7 engagement (8) was confirmed with both T cell lymphoma EL4 cells (Fig. 2A) and WT lymph node CD4 cells (Fig. 2B). IL-7Rα was detectable on the surface of these cells by confocal microscopy in the absence of IL-7 (top row, panel I of Figs. 2A and 2B), and was located at a similar position as cell surface Thy1.2 or CD4 (top row, panels II and III of Figs. 2A and 2B). After 3 h of IL-7 exposure, Thy1.2 and CD4 remained on the cell surface (middle row, panel II, Figs. 2A and 2B), but IL-7Rα, being still detectable, was largely internalized and moved into the cytoplasm inside Thy1.2 or CD4 circles (middle row, panels II and III of Figs. 2A and 2B). At the same time, IL-7Rα expression was drastically reduced on the surface of EL4 cells and spleen CD4 cells (bottom row, Fig. 2A and 2B) upon IL-7 stimulation, while Th1.2 and CD4 expression on these cells remained unchanged in the absence or presence of IL-7, according to flow cytometry, further proving that IL-7-triggered IL-7Rα internalization in these cells.

We further demonstrated that after receiving IL-7 stimulation, WT naïve CD4 T cells ($CD44^{lo}CD62L^{hi}$) showed a time-dependent reduction (assayed at 20, 50 and 90 min) of IL-7Rα on the cell surface (Fig. 3A, left panel), mainly due to its internalization, as described above. EFNb1 and EFNb2 deletion (i.e., dKO) in these cells resulted in a greater rate of IL-7Rα reduction (Fig. 3A, right panel), while IL-7Rα mRNA level was not affected by the absence of EFNb1 and EFNb2, or the presence of IL-7 (Fig. 3B) within the 90-min period, indicating that IL-7Rα down-regulation in CD4 cells upon IL-7 stimulation occurs at the post-translational level in this period.

We wondered whether the opposite was also true: i.e., EFNb1 and EFNb2 over-expression would retard IL-7Rα down-regulation upon IL-7 stimulation. For this purpose, T cell lymphoma EL4 cells

were stably transfected with the EFNb1 and EFNb2 expression constructs pCEP-EFNb1 or pCEP-EFNb2, respectively. EFNb1 and EFNb2 over-expression on the cells was confirmed by flow cytometry (Supplementary Fig. 1A), and the cells were named EFNb1-EL4 and EFNb2-EL4, respectively. Their cell surface IL-7Rα down-regulation upon IL-7 exposure was significantly retarded, when compared to EL4 cells transfected with empty vector (vector-EL4; Fig. 3C). On the other hand, IL-7Rα mRNA levels in EFNb1-EL4 and EFNb2-EL4 cells were comparable to vector-EL4 cells, in the absence or presence of IL-7 (Fig. 3D), confirming that IL-7Rα down-regulation is at the post-transcriptional level during this time frame. Taken together, these findings confirm that the presence of EFNb1 and EFNb2 retards IL-7Rα down-regulation on the T cell surface upon IL-7 stimulation; conversely, the absence of EFNb1 and EFNb2 accelerates IL-7-triggered IL-7Rα down-regulation.

In contrast to IL-7Rα, EFNb1 and EFNb2 expression after anti-EFNb1 or EFNb2 Ab cross-linking (Supplementary Figs. 1B and 1C, middle row) or IL-7 treatment (bottom row) remained largely unchanged, compared to cells cultured in plain medium (upper row).

EFNb1 and EFNb2 physically interact with IL-7Rα and anchor it on the cell surface

We then questioned whether EFNb1 and EFNb2 associated physically with IL-7Rα as a mechanism to reduce IL-7Rα internalization. When EFNb1-EL4 cells were cross-linked with anti-EFNb1 Ab plus anti-IL-7Rα Ab, both EFNb1 and IL-7Rα formed caps within 10 min before IL-7Rα internalization occurred. EFNb1 and IL-7Rα co-localized in the cap on the cell surface (Fig. 4A, left panel), whereas EFNb1 and Thy1.2 did not co-cap when EFNb1-EL4 cells were cross-linked with anti-EFNb1 Ab plus anti-Thy1.2 Ab (Fig. 4A, right panel). Similarly, EFNb2 and IL-7Rα co-capping also manifested in EFNb2-EL4 cells when cross-linked with anti-EFNb2 Ab plus anti-IL-7Rα Ab (Fig. 4B, left panel), but EFNb2 and Thy1.2 did not co-cap when EFNb2-EL4 cells were cross-linked with anti-EFNb2 plus anti-Thy1.2 Ab (Fig. 4B, right panel). The co-migration of EFNb1 or EFNb2 with IL-7Rα raises the possibility that EFNb1 and EFNb2 may directly associate with IL-7Rα on the T cell surface.

It should be noted that if only 1 Ab (i.e., anti-IL-7Rα or anti-EFNb1 Ab) was used in the cross-linking step, IL-7Rα and EFNb1 (or EFNb2) would still undergo discernible but faint co-capping between EFNb1 and IL-7Rα, or between EFNb2 and IL-7Rα, respectively, under the microscope, but the

resulting images were not satisfactory for illustration. This was mainly due to a technical difficulty: the cells needed to be fixed to terminate cross-linking, but the other Ab (the one not deployed in initial cross-linking) could not stain fixed cells well as it would fresh cells, while in double cross-linking experiments (Fig. 4), both Abs (i.e., anti-IL-7Rα and anti-EFNb1 Ab) reacted with fresh cells.

Two methods of fluorescence resonance energy transfer (FRET), i.e., acceptor photo bleaching (AB) and sensitized emission (SE), were employed to assess such a possibility. Figures 5A and 5B show FRET from IL-7Rα to EFNb1 and from IL-7Rα to EFNb2, respectively, using AB. In both figures, panel I presents micrographs illustrating immunofluorescence staining of IL-7Rα versus EFNb1 in EFNb1-EL4 cells, and IL-7Rα versus EFNb2 in EFNb2-EL4 cells. The upper half of the figures illustrates EFNb1-EL4 cells or EFN2-EL4 cells without cross-linking of their IL-7Rα or EFNbs. EFNb1, EFNb2 and IL-7Rα were distributed rather uniformly on the cell surface before AB. After AB, acceptor (EFNb1 or EFNb2) but not donor (IL-7Rα) signals were drastically weakened, proving the effectiveness of acceptor-specific bleaching. The lower half of the figures show EFNb1-EL4 or EFNb2-EL4 cells cross-linked with anti-EFNb1/anti-IL-7Rα Abs or anti-EFNb2/anti-IL-7Rα Abs, respectively. In these cells, EFNb1 or EFNb2 co-capped with IL-7Rα; AB depleted EFNb1 and EFNb2 but not IL-7Rα signals. The donor fluorescence intensity of selected areas (in colored circles) in the bleached region (rectangles) and control areas outside the bleached region of sample cells (panel II, Figs. 5A and 5B) was quantified, and data from these representative cells are listed in the tables in panel III of Figs. 5A and 5B. In both EFNb1-EL4 cells (table in panel III of Fig. 5A) and EFNb2-EL4 cells (table in panel III of Fig. 5B), without (upper half of the tables) or with cross-linking (lower half of the tables), there was a bigger increase in donor (IL-7Rα) fluorescence intensity in the area inside the AB region, compared to the area outside the bleached region. The means ± SD of FRET efficiency (from IL-7Rα to EFNb1 or from IL-7Rα to EFNb2) of areas inside and outside the bleached regions of more than 10 randomly selected cells are shown in panel IV of Figures 5A and 5B. The results indicate that IL-7Rα associates with EFNb1 and EFNb2 constitutively and after IL-7Rα engagement.

The results based on AB FRET were verified with SE FRET. As illustrated in Figure 6A, FRET efficiency based on energy transfer from IL-7Rα to EFNb1 or from IL-7Rα to EFNb2 was significantly higher than that of the controls (i.e. FRET efficiency from Thy1.2 to EFNb1 or from

Thy1.2 to EFNb2), whether IL-7Rα/EFNb1 or IL-7Rα/EFNb2 was cross-linked (Fig. 6A, right panel) or not (Fig. 6, left panel). It should be noted that SE FRET efficiency in both controls was near-null.

Immunoprecipitation was performed to further prove the physical interaction between EFNb1/EFNb2 and IL-7Rα. CHO cells were first stably transfected with Myc-tagged EFNb1 or EFNb2, and then transiently transfected with HA-tagged IL-7Rα. The over-expression of Efbn1, EFNb2 (Supplementary Fig. 2A) and IL-7Rα (Supplementary Fig. 2B) in these cells was confirmed by flow cytometry. In anti-Myc immunoprecipitation of CHO cells with EFNb1-Myc/IL-7Rα–HA double transfection or EFNb2-Myc/IL-7Rα–HA double transfection (Fig. 6B, first and last lanes), IL-7Rα–HA could be detected by anti-HA Ab in immunoblotting. No HA signals were detected in CHO cells with IL-7Rα–HA single transfection (Fig. 6B, middle lane), excluding the possible carry-over of IL-7Rα -HA by anti-Myc Ab-conjugated beads. The presence of EFNb1-Myc and EFNb2-Myc in the first and last lanes was confirmed by anti-Myc Ab immunoblotting (Fig. 6B, lower panel).

With FRET and immunoprecipitation, we thus demonstrated that IL-7Rα directly associates with EFNb1 and EFNb2.

II.4.4 DISCUSSION

In the present study, we discovered that EFNb1 and EFNb2 physically interact with IL-7Rα, and modulate its internalization and signaling, with functional consequences.

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

In contrast to IL-7Rα, EFNb1 and EFNb2 expression levels on the cell surface are not subjected to modulation by IL-7 engagement, nor are these 2 molecules internalized after being engaged by their agonists. The association between EFNb1/EFNb2 and IL-7Rα thus enables the former to act as anchors to moor IL-7Rα on the cell surface and prevent it from rapid internalization. In the absence of EFNb1 and EFNb2, this mooring effect is lost. Ex vivo examination of thymocytes and peripheral T cells disclosed that IL-7Rα levels on these cell surfaces are reduced, likely due to faster internalization caused by ambient or stimulated IL-7 in vivo. It should be noted that the mooring effect of EFNb1 and EFNb2 is additive, as the reduction of IL-7Rα in thymocytes and T cells only becomes apparent when both, but not only one of them, are deleted.

The effect of EFNb1 and EFNb2 on IL-7-stimulated IL-7Rα down-regulation on the cell surface seems to have certain specificity. IL-7Rα on T cells is also down-regulated after TCR stimulation (22), and this is not an IL-7-dependent event. We showed that such down-regulation occurred at a slower pace compared to IL-7-induced down-regulation, only being apparent 16 h after TCR stimulation and not influenced by a lack of EFNb1 and EFNb2 (supplementary Fig. 3A, left panel). An explanation is that such down-regulation is mainly at the transcription level (Supplementary Fig. 3B, right panel), which is not subjected to the EFNb1 and EFNb2 anchoring effect on the cell surface. The non-promiscuous nature of the EFNb1/EFNb2 effect was also demonstrated in the case of IL-6Rα. IL-6Rα on T cells are also internalized after encountering IL-6 (23), but this event was not affected by EFNb1 and EFNb2 deletion (Supplementary Fig. 3B). With that said, it is still possible that EFNb1 and EFNb2 anchor cell surface receptors other than IL-7Rα and modulate their internalization.

The anchoring effect of EFNb1 and EFNb2 on IL-7Rα was not affected by EFNb1 and EFNb2 engagement, as the rate of IL-7Rα internalization upon IL-7 stimulation in EFNb1-EL4 and EFNb2-EL4 cells was not influenced by solid phase anti-EFNb1 and anti-EFNb2 Ab in culture wells (bottom row, Supplementary Fig. 4), when compared with those cultured in uncoated wells (top row, Supplementary Fig 4).

In the absence of EFNb1 and EFNb2, IL-7Rα expression was down-regulated, likely as a consequence of enhanced internalization. What is the functional outcome of such increased IL-7Rα down-

regulation? A recent report suggests that IL-7Rα internalization is necessary for IL-7R signaling, because treating leukemic cells with hyperosmotic sucrose, which inhibits IL-7Rα internalization, results in reduced JAK1/3, STAT5 and AKT signaling (8). It follows that increased IL-7Rα internalization that causes decreased IL-7Rα expression should promote IL-7R signaling. The validity of such a conclusion is debatable, primarily because hyperosmotic treatment disturbs the entire cell biology, including multiple signaling pathways. A more intuitive conclusion is that reduced IL-7Rα expression in the T cell compartment evoked by increased internalization will lead to compromised IL-7-dependent thymocyte and T cell functions. Several functional studies support such a conclusion. We presented evidence that STAT5 phosphorylation, a critical IL-7R signaling event, was abated in dKO CD4 cells after IL-7 stimulation. As shown in Figure 1D, dKO CD4 and CD8 cells manifested compromised in vivo homeostatic proliferation, an IL-7-dependent event. Therefore, our data indicate that one of the physiological functions of EFNb1 and EFNb2 is to stabilize IL-7Rα expression and enhance IL-7 signaling in the T cell compartment. Under physiological conditions, the modulation of such stabilization probably depends on the EFNb1 and EFNb2 expression level.

The finding that EFNbs directly associate with other receptors and influence their stability at the cell surface reveals a previously-unknown *modus operandi* of EFN. It is conceivable that EFNs other than EFNb1 and EFNb2 might also have such a capability, and they might affect not only IL-7Rα stability and function but also other receptors or cell surface molecules. In a broader sense, such effects could be viewed as part of EFN "reverse signaling," as this imparts changes of signaling into the cells via EFNs, although such "reverse signaling" does not necessarily need to be triggered by Ephs, and is not necessarily transduced directly via EFNs, but via the receptors they associate with. Recently, Sawamiphak et al. (24) and Wang et al. (25) reported that, in endothelial cells, EFNb2 facilitates VEGFR2 and VEGFR3 internalization, which, for VEGFRs, is an essential step for their signaling. Wang et al. observed rapid cell surface co-localization of EFNb2 and VEGFR3 on cells upon VEGF-C stimulation (25). Also, EFNb2 physically binds to VEGFRs according to immunoprecipitation (25), an additional example of EFNb modulating the function of other receptors at the post-translational level. It seems that whether such interaction retards or enhances the internalization of other molecules is case-dependent; factors that determine whether internalization is reduced or enhanced remain to be elucidated.

Most documented functions of Ephs and EFNbs are related to pattern formation, and depend on interaction between Ephs and EFNs on neighboring cells. The findings of our current study on the effect of EFNbs on post-translational modulation of IL-7Rα in the T cell compartment have revealed EFNb functions that have little to do with pattern formation. The mooring effect of EFNbs on IL-7Rα does not seem to require direct input from Ephs, although it is conceivable that factors modulating EFNb1 and EFNb2 expression will in turn alter IL-7Rα expression. Such a novel *modus operandi* of EFNb1 and EFNb2 in regulating the functions of other cell surface receptors at the post-translational level has revealed a previously under-appreciated regulatory role between different cell surface receptors

II.4.5 ACKNOWLEDGMENTS

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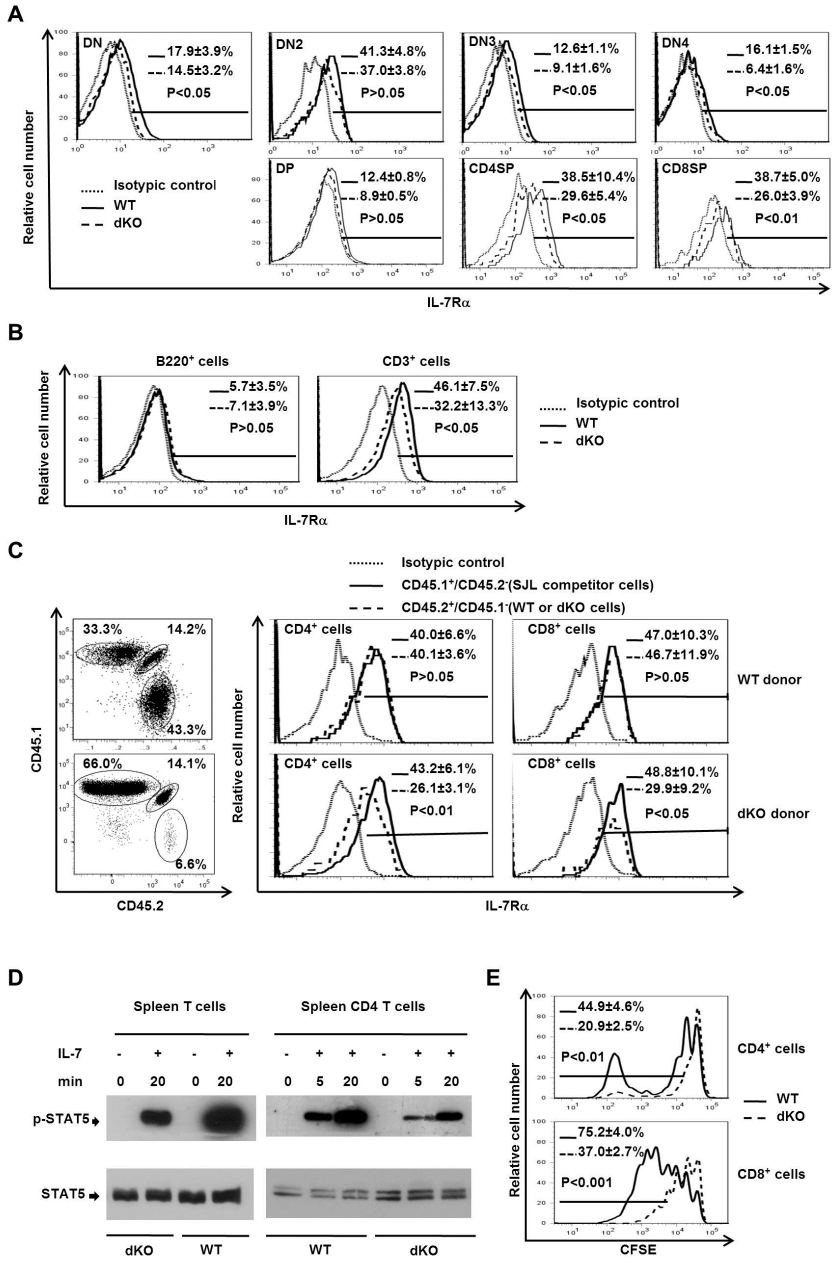
II.4.7 FIGURES and LEGENDS

Figure 2.20. Reduced IL-7Rα expression in dKO thymocytes and T cells



All experiments in this figure were repeated 3 times or more, and representative results are reported. Flow cytometry histograms show the mean + SD of percentages of IL-7Ra-positive cells (A, B and C) and the percentages of cells with reduced CFSE (E) from all repetitions. p values (Student's t test) are indicated.

A. IL-7Ra expression in DN, DN2, DN3, DN4, DP, CD4SP and CD8SP thymocytes.

Thymocytes were gated on DN, DN2, DN3, DN4, DP, CD4SP or CD8SP cells, and their IL-7Ra expression was analyzed by flow cytometry.

B. IL-7Ra expression in T and B cells.

Spleen cells were gated on B220+ B cells or CD3+ T cells, and their IL-7Ra expression was analyzed by flow cytometry.

C. Reduced IL-7Ra expression in spleen CD4 and CD8 T cells derived from dKO bone marrow cells in irradiated recipients.

T cell-depleted dKO (lower row) and WT (upper row) bone marrow cells (CD45.2+) 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 x B6.SJL F1 recipients. After 60 days, spleen CD4 and CD8 cells were analyzed by flow cytometry. CD45.2+ cells were derived from dKO or WT bone marrow, CD45.1+ cells were from competing B6.SJL bone marrow cells, and CD45.1+/CD45.2+ cells were from residual cells of the recipients. CD4 and CD8 cells from CD45.2+ or CD45.1+ spleen cells were gated and analyzed for IL-7Ra expression.

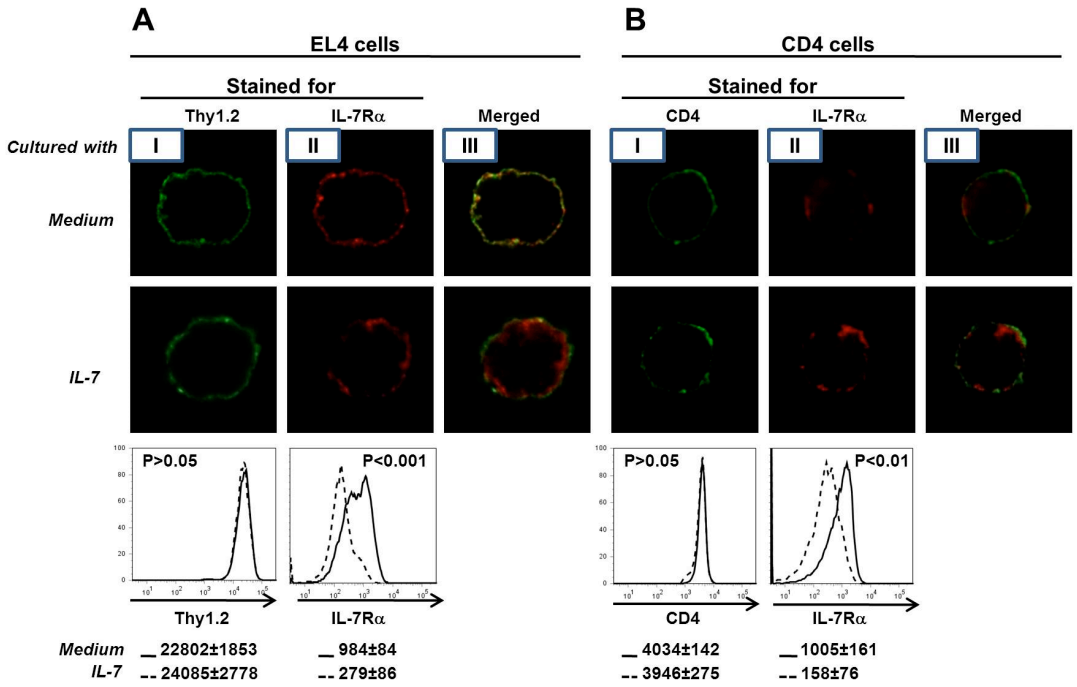
D. Reduced STAT5 phosphorylation in dKO T cells upon IL-7 stimulation.

dKO and WT spleen T cells or CD4 cells were stimulated with IL-7 (10 ng/ml) at 37oC for the periods indicated, and then lysed. Phosphorylated STAT5 (p-STAT5) in lysates was assessed by immunoblotting. Total STAT5 protein in lysates was evaluated by re-blotting the same membrane with anti-STAT5 Ab.

E. dKO T cells presented failed homeostatic expansion in sub-lethally irradiated recipients.

B6.SJL mice (CD45.1+) were sub-lethally irradiated at 600 Rads and transplanted i.v. with 4 x 106 CFSE-labeled spleen cells from dKO or WT mice (both CD45.2+). The histograms represent profiles of CFSE-positive cells gated on CD4+CD45.2+ and CD8+CD45.2+ cells.

Figure 2.21. IL-7Rα internalization in EL-4 cells and spleen CD4 T cells.

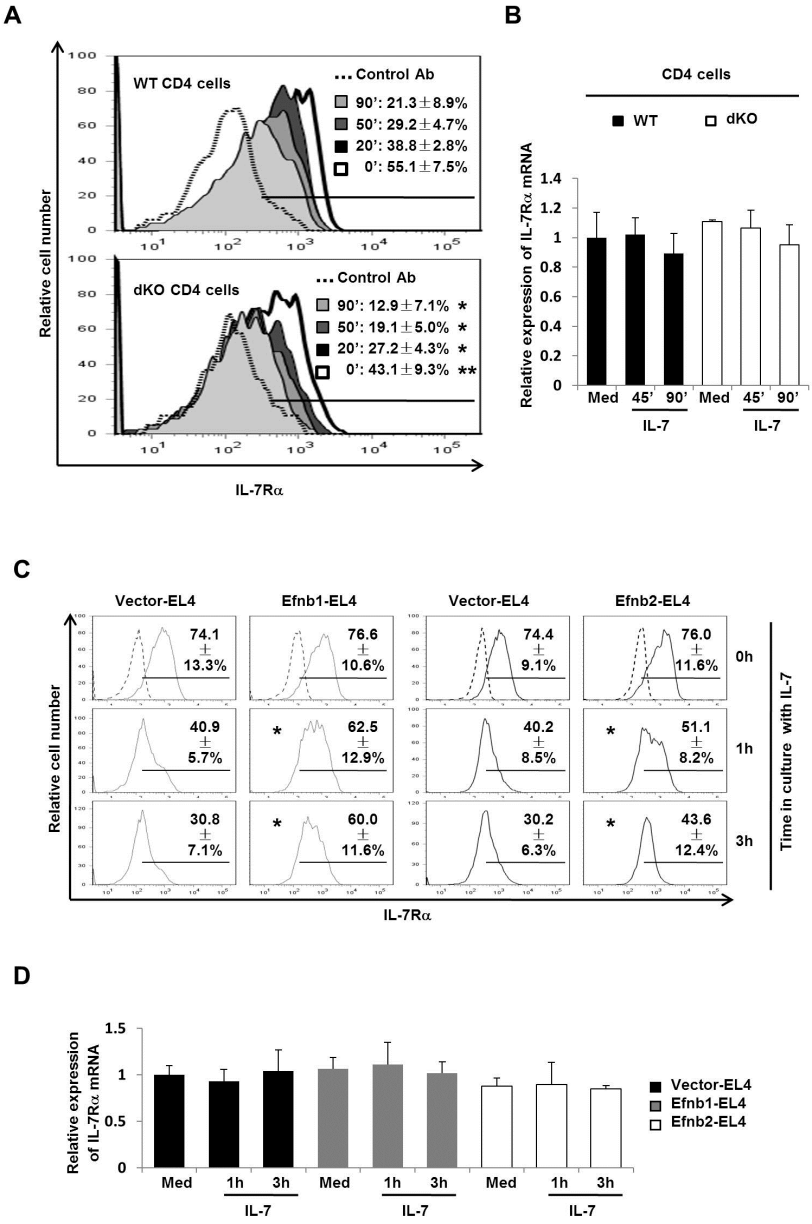


EL4 cells (A) were cultured in medium without IL-7 (top row) or with IL-7 (20 ng/ml, middle row) for 3 h, washed and stained with FITC-anti-Thy1.2 mAb. WT naïve CD4 cells (B) from lymph nodes were first activated with soluble anti-CD3ε and anti-CD28 mAb for 48 h to increase cell cytoplasm content for better visualization during imaging. The cells were left to rest for 4 days. These activated cells were cultured in medium without IL-7 (top row) or with IL-7 (20 ng/ml, middle row) for 3 h, washed and stained with FITC-anti-CD4 mAb.

The cells were then fixed, permeabilized and stained with biotinylated anti-IL-7Rα, followed by Alexa Flour-568-conjugated streptavidin. Fluorescent signals were detected with confocal microscopy. Merged images (panel III) show the position of IL-7Rα (in red) relative to cell surface Thy1.2 (A, in green) or CD4 (B, in green). The experiments were repeated more than 3 times and representative data are reported.

The cells were also stained for IL-7Rα plus Thy1.2 or CD4 without permeabilization and analyzed by flow cytometry (bottom row). Solid line: cells cultured in medium; dotted line: cells cultured in the presence of IL-7. The means $\pm$ SD of fluorescence intensity in more than 3 experiments are indicated in the histograms, and p values are presented (Student's t test).

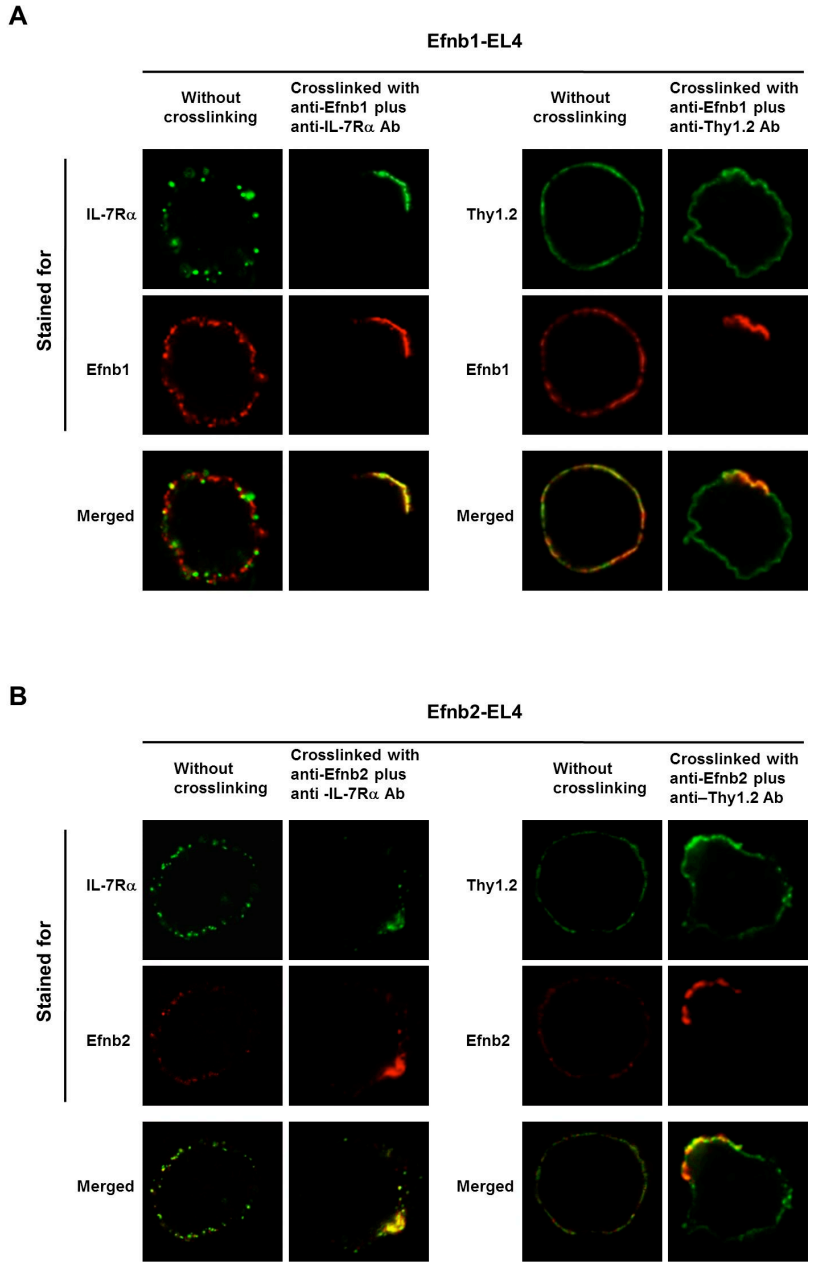
Figure 2.22. EFNb1 and EFNb2 expression modulates IL-7Ra expression on the cell surface.



All experiments in this figure are repeated 3 times or more, unless stated otherwise. Representative data are shown. Mean + SD of all the repetitions are indicated in the histograms. P values are indicated by * (Student's t tests).A. Faster IL-7-induced IL-7Rα decrease on the surface of dKO CD4 cells.Lymph node naïve CD4 cells from dKO or control WT mice were cultured in the presence of IL-7 (20 ng/ml). Their cell surface IL-7Rα was quantified by flow cytometry at different time points (0 min, 50 min and 90 min) post-IL-7 addition. The percentages of IL-7Rα-positive cells are indicated. * and ** indicate p values less than 0.05 and 0.01, respectively, comparing parameters of the same time point.B. IL-7Rα mRNA levels in dKO CD4 cells remained stable for 90 min after IL-7 stimulation. Naïve CD4 T cells from dKO or WT mice were cultured in plain medium (Med) or in the presence of IL-7 (20 ng/ml) for

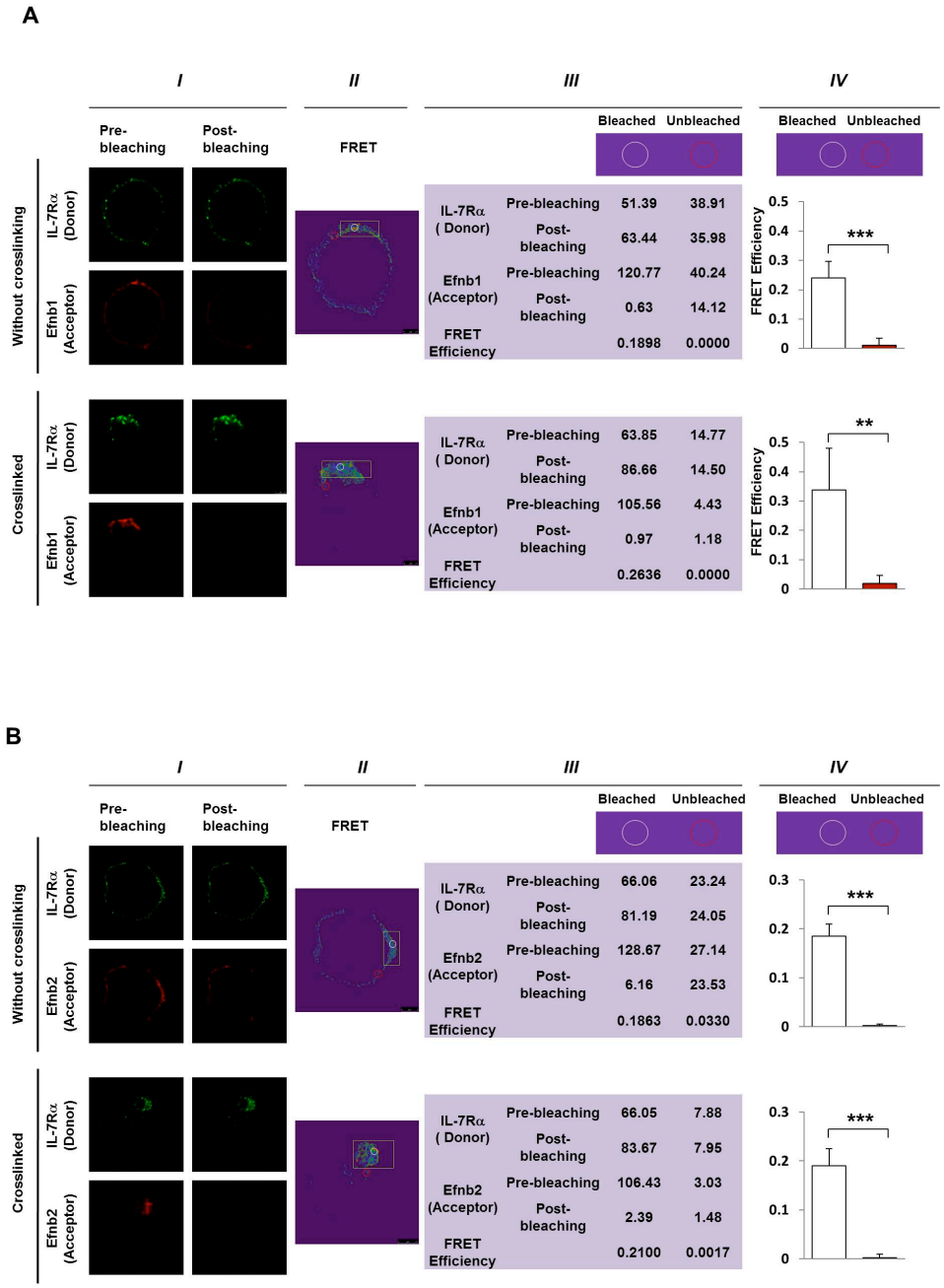
45 min or 90 min. IL-7Rα mRNA of these cells was measured by RT/qPCR. The ratios of IL-7Rα/β-actin signals were obtained first. The data were then normalized and presented as the mean ± SD of relative IL-7Rα expression, with the ratio from WT CD4 cells cultured in medium being considered as 1 unit. C. Slower IL-7-induced decrease of IL-7Rα on the surface of EFNb1-EL4 and EFNb2-EL4 cells compared to vector-transfected EL4 cells.
EFNb1-expressing EL4 cells (EFNb-EL4), EFNb2-expressing EL4 cells (EFNb2-EL4) and vector-transfected EL4 cells (vector-EL4) were cultured in the absence or presence of IL-7 (20 ng/ml). Their cell surface IL-7Rα were quantified by flow cytometry at different time points (0 h, 1 h, 3 h) post-IL-7 addition. * indicates p values less than 0.05 between EFNb1-EL4 and vctor-EL4, or between EFNb2-EL4 and vector EL4. D. IL-7Rα mRNA levels in EFNb1-EL4 and EFNb2-EL4 cells remained stable for 3 h after IL-7 stimulation.IL-7Rα mRNA of EFNb1-EL4 or EFNb2-EL4 cells cultured in medium (Med) or in the presence of IL-7 (20 ng/ml) for 1 h and 3 h was measured by RT/qPCR. The ratios of IL-7Rα/β-actin signals were obtained first. The data were then normalized and presented as the mean ± SD of relative of IL-7Rα expression, with the ratio from vector-EL4 cells cultured in medium being considered as 1 unit.

Figure 2.23. IL-7Rα co-localizes with EFNb1 and EFNb2 after IL-7Rα/EFNb1 or IL-7Rα/EFNb2 cross-linking.



EFNb1-EL4 cells (A) and EFNb2-EL4 cells (B) were cultured in plain medium (without cross-linking), or cross-linked with anti-EFNb1 plus anti-IL-7Rα Ab, anti-EFNb2 plus anti-IL-7Rα Ab, anti-EFNb1 plus anti-Thy1.2 Ab, or anti-EFNb2 anti-thy1.2, as indicated. IL-7Rα, Thy1.2, EFNb1 and EFNb2 expression on the cell surface was detected by confocal microscopy.

Figure 2.24. EFNb1/EFNb2 associate with IL-7Rα.according to AB FRET.

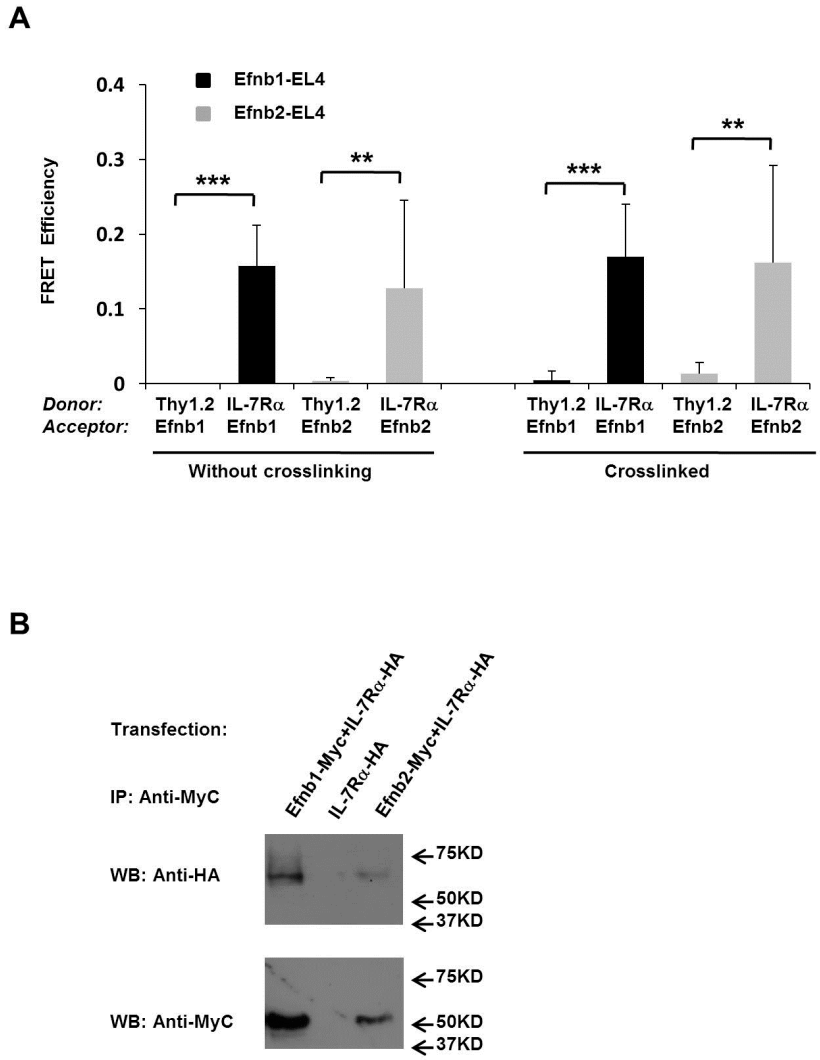


EFNb1-EL4 cells (A) and EFNb2-EL4 cells (B) were analyzed for interaction between EFNb1 and IL-7Rα, and between EFNb2 and IL-7Rα, respectively, by AB FRET. The cells were not Ab-cross-linked (upper half of the figures) or Ab-cross-linked (anti-IL-7Rα Ab plus anti-EFNb1 Ab in A, and anti-IL-7Rα Ab plus anti-EFNb2 Ab in B; lower half of the figures).

Panel I of A and B shows IL-7Rα (donor fluorophore in AB FRET) expression and EFNb expression (acceptor fluorophore in AB FRET) before and after AB. Panel II of A and B illustrates the AB region (in rectangle) and selected areas (in colored circles) of a sample cell for AB FRET analysis. Panel III of A and B presents tables containing fluorescence intensity of a bleached area (white circle) and

unbleached area (red circle) in the sample cell of panel II. Panel IV presents bar graphs summarizing AB FRET efficiency based on data from more than 10 randomly-selected cells (2 circles per cell inside the bleached area and 2 circles per cell outside the bleached area).

Figure 2.25. Interaction between EFNb1/EFNb2 and IL-7Rα according to SE FRET and immunoprecipitation.

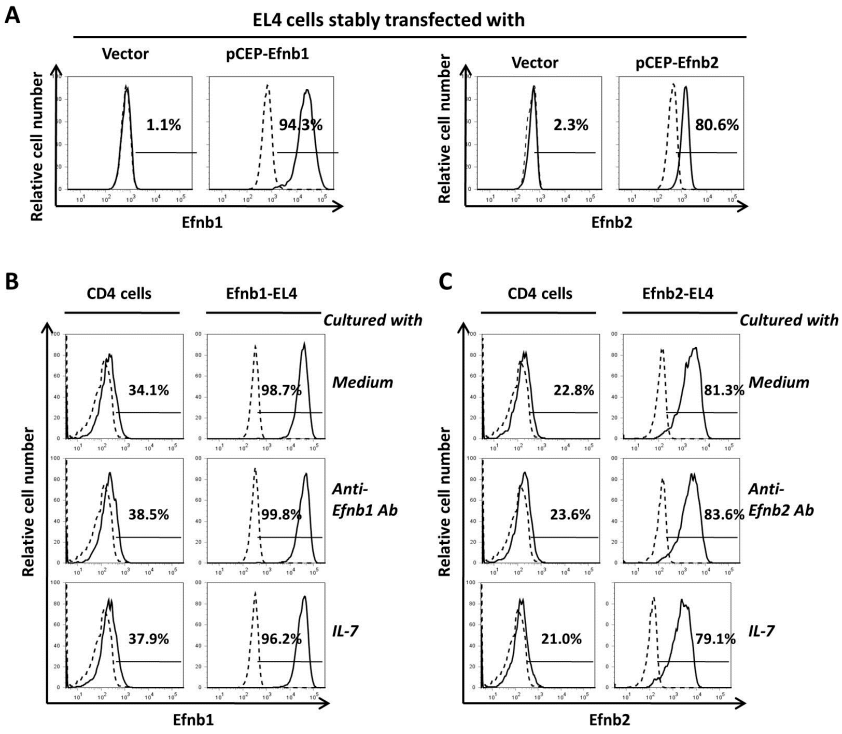


A. SE FRET. EFNb1-EL4 cells and EFNb2-EL4 cells were cross-linked with a pair of Abs (anti-IL-7Rα or anti-Thy1.2 Ab plus anti-EFNb1 Ab for EFNb1-EL4 cells, and anti-IL-7Rα or anti-Thy1.2 Ab plus anti-EFNb2 Ab for EFNb2-EL4 cells), or remained untreated, as indicated. Mean ± SD of SE FRET efficiency between IL-7Rα and EFNb1, between IL-7Rα and EFNb2, between Thy1.2 and EFNb1, and between Thy1.2 and EFNb2 are presented. ** and *** indicate $p<0.01$ and $p<0.001$, respectively (Student's t test). B. Immunoprecipitation. CHO cells stably expressing Myc-tagged EFNb1 or EFNb2 were transiently transfected for HA-tagged IL-7Rα expression. The cell lysates were precipitated with anti-Myc Ab coated agarose beads. The proteins were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes, which were sequentially blotted with anti-HA Ab and anti-Myc Ab. IP: immunoprecipitation; WB: Westen blotting; EFNb1-Myc + IL-7Rα-HA: CHO cells stably expressing Myc-tagged EFNb1 were transiently transfected for HA-tagged IL-7Rα expression; EFNb2-Myc + IL-7Rα-HA: CHO cells stably expressing Myc-tagged EFNb2 expression were transiently transfected for HA-tagged IL-7Rα expression; IL-7Rα-HA: WT CHO cells were transiently transfected for IL-7Rα expression as negative control for immunoprecipitation.

All experiments in this figure were repeated twice or more, and representative cells or data are reported.

II.4.8 SUPPLEMENTARY FIGURE LEGENDS

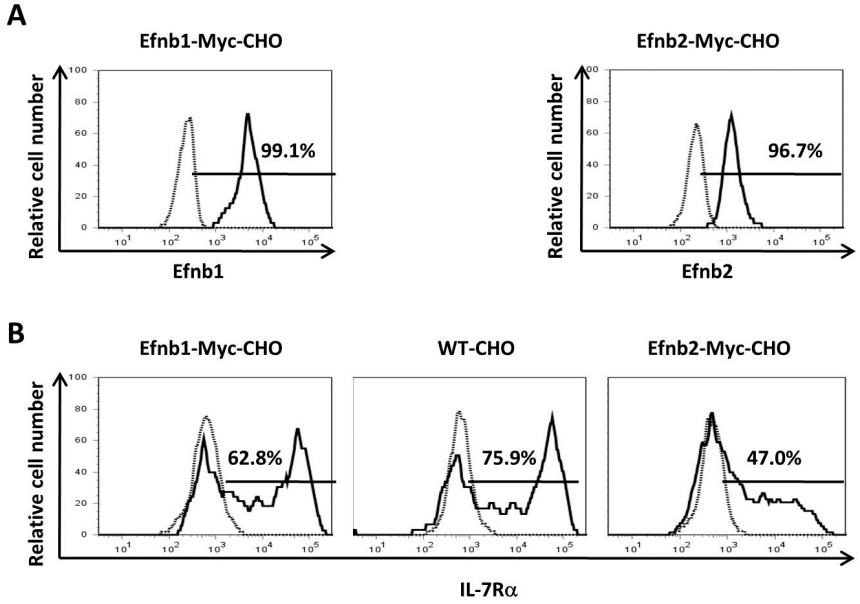
Supplementary Figure 1. Stable EFNb1 and EFNb2 expression in EL4 and CD4 cells upon anti-EFNb Ab or IL-7 stimulation.



A. EFNb1 and EFNb2 over-expression in EL4 cells. EL4 cells were stably transfected with empty pCEP4 vector (Vector), pCEP-EFNb1 or pCEP-EFNb2 by electroporation. The cell surface EFNb1 and EFNb2 expression of these cells was analyzed by flow cytometry. Percentages of positively stained cells (solid line) are indicated after subtracting percentages of control Ab (goat IgG)-stained cells (dotted line). B and C. EFNb1 and EFNb2 expression on the cell surface not affected by anti-EFNb1 and anti-EFNb2 Abs or by IL-7. Naïve spleen CD4 cells (left column of B and C) from WT mice, or EFNb1-EL4 cells or EFNb2-EL4 cells (right column of B and C) were cultured for 3 h in medium (upper row), or in the presence of soluble anti-EFNb1 or anti-EFNb2 Ab (5 μg/ml; middle row), or in the presence of IL-7 (20 ng/ml; bottom row), as indicated. The cell surface expression of EFNb1 and EFNb2 was assessed by flow cytometry, and percentages of positive cells are reported.

All experiments in this figure were repeated more than twice, and representative data are reported.

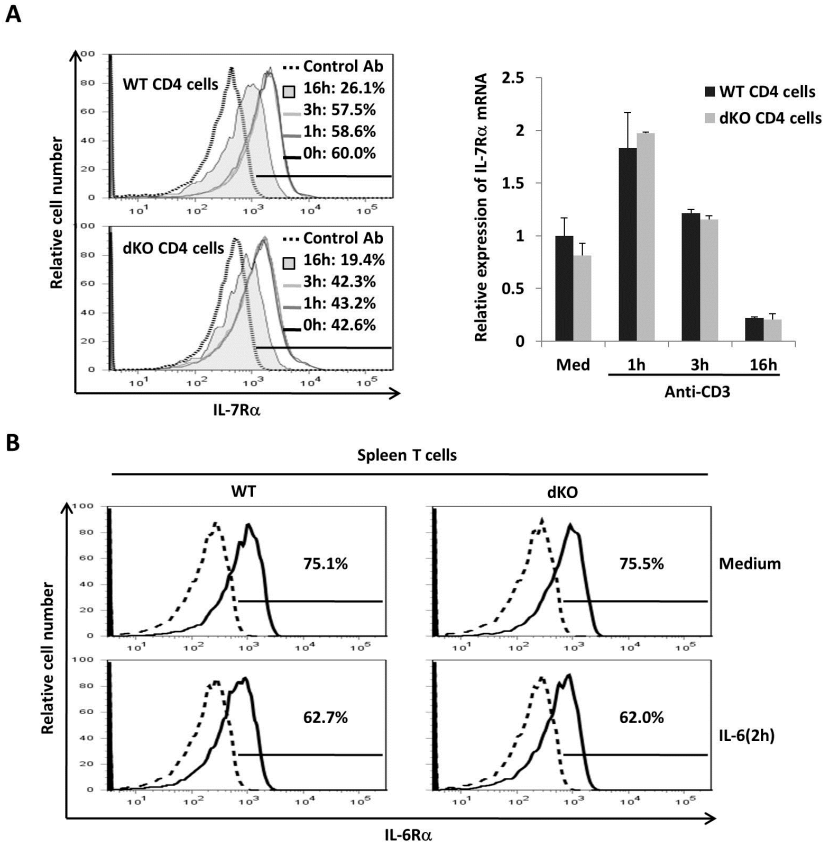
Supplementary Figure 2. Stable Myc-tagged EFNb1 and EFNb2 expression and transient HA-tagged IL-7Rα expression in CHO cells according to flow cytometry.



A. EFNb1 and EFNb2 over-expression. CHO cells were stably transfected with p-Receiver-EFNb1-Myc or pReceiver-EFNb2-Myc, and their cell surface EFNb1 or EFNb2 over-expression was assessed by flow cytometry. Dashed line: WT CHO cells; solid line: stably transfected CHO cells.B. IL-7Rα over-expression. Stably transfected CHO cells, as described in A, or WT CHO cells were transiently transfected with p-Receiver-IL-7Rα-HA, and their IL-7Rα over-expression was assessed by flow cytometry 30 h after transfection. Dashed line: CHO cells (WT or stably transfected) without p-Receiver-IL-7Rα-HA transient transfection; solid line: CHO cells (WT or stably transfected) with p-Receiver-IL-7Rα-HA transient transfection.

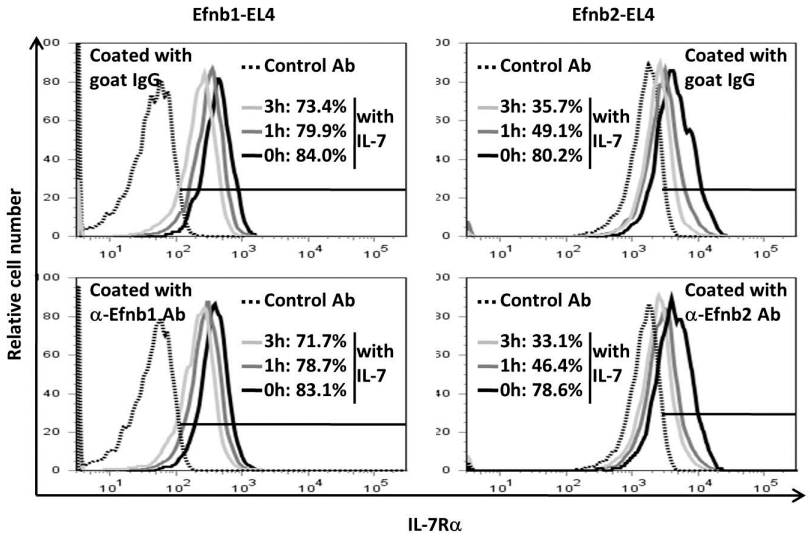
All experiments in this figure were repeated more than twice, and representative data are reported.

Supplementary Figure 3. Lack of effect of EFNb1 and EFNb2 on the expression of TCR activation-induced IL-7Rα down-regulation and IL-6-induced IL-6Rα down-regulation.



A. EFNb1 and EFNb2 deletion had minimal effect on TCR activation-induced IL-7Rα down-regulation. Spleen CD4 cells from dKO or WT mice were stimulated with solid phase anti-CD3ε (4 μg/ml during coating). The cells were harvested at different time points as indicated, and their IL-7Rα expression was assessed by flow cytometry. Percentages of IL-7Rα$^+$ cells are indicated in the histograms (left panel). The IL-7Rα mRNA levels of these cells were measured by RT-qPCR and results are expressed as Mean + SD of the ratios of IL-7Rα/β-actin signals (right panel). B. EFNb1 and EFNb2 deletion had minimal effect on IL-6-induced IL-6Rα down-regulation. Spleen T cells from dKO or WT mice were cultured in medium or stimulated with IL-6 (50 ng/ml) for 2 h. Their IL-6Rα expression was assessed by flow cytometry. Percentages of IL-6Rα$^+$ cells are indicated in the histograms. All experiments in this figure were repeated more than twice, and representative data are reported.

Supplementary Figure 4. EFNb1 and EFNb2 engagement does not affect IL-7Rα down-regulation.



EFNb1-EL4 and EFNb2-EL4 cells were cultured in wells coated with anti-EFNb1 and EFNb2 Abs (4 μg/ml during coating) for 24 h, and treated with IL-7 (20 ng/ml) for periods as indicated. Their IL-7Rα expression was assessed by flow cytometery. Percentages of IL-7Rα$^+$ cells are indicated in the histograms.

III. DISCUSSION

III.1 Discussion

EFNs are the ligands of the receptor tyrosine kinases, Eph, which constitute the largest family of receptor tyrosine kinases. EFN and Ephs are widely expressed in various tissues and exert critical functions such as neuron axon guidance, angiogenesis, skeletal development, and tissue patterning.[2, 3] In this study, we aim to elucidate the function of EFNB1, EFNB2 and EphB4 in the immune system from different angles. In article 1 and 2, we specifically deleted *EFNB1* and *EFNB2* in T cells using LoxP/cre system. The cre recombinase used in these two studies is driven by proximal Lck promoter (referred as lck-cre in following context). The Lck-cre is activated in thymocytes starting from the DN3 stage, which deletes the loxp flanked *EFNB1* or *EFNB2* gene in thymocytes from the DN3 stage and onwards. [277]

Mice lacking either EFNB1 or EFNB2 demonstrated mild abnormalities in T cell populations with an elevated DN population. Within the DN stage, we observed an arrest of progression from the DN3 to DN4 stage. On the other hand, the function of mature T cells with regard to T cell activation as well as their differentiation potential to different T-helper lineages is not influenced. One might argue that the mild phenotype may reflect the fact that EFNB1 and EFNB2 only play a minor role in T cell development. However, our observation, on the contrary, suggests an essential role of EFNB1 and EFNB2. Interaction between Ephs and EFNs leads to activation of both forward and reverse signalling respectively into their host cells. [2] The thymus consists of a tightly packed structure, in which migrating thymocytes consistently interact with surrounding TECs and thymocytes. [273] Therefore, developing thymocytes receive both forward and reverse signalling from TEC as well as neighbouring thymocytes. Our early study has shown that interfering with the bindings of EFNB1 to its receptors causes a significant thymic subpopulation ratio skew. [89] In peripheral T cells, expression of all three members EFNBs as well as their receptors has been reported.[91-93] Activating EphBs forward signalling using solid phase EFNB1 co-stimulates T cell activation and modulates responses of activated $CD4^+$ T cells. Similar findings also apply to EFNB2 and EFNB3. [91, 92] EFNB1 and EFNB2 share a high sequence homology, [4] and theyhave lose specificity to multiple Eph receptors, such as EphB2, EphB4 as well as EphB6. [278] Therefore, each EFNB is capable of activating several Eph receptors, indicating their functional redundancy in the T cell compartment. Our later research demonstrate that EFNB1 and EFNB2 double knockout in T cells leads to multiple defects of development and function in T cells

compartment. [40] Therefore, it is reasonable to argue that the role of EFNB1 and EFNB2 in immune system is so critical that, if there were only one of them, accidental mutation could lead to severe consequences. So we have developed a heavily redundant Eph and EFN system through evolution as a safe guard. Combining with the promiscuous binding pattern of Eph/ EFN, such a system could compensate the lose-of-function mutation happening to a single family member in the T cell compartment.

Thymocytes undergo negative selection and final maturation in the thymic medulla. During the process, mTECs play a key role in establishing central tolerance. [40] Our previous research has revealed the important role of EFNB1 and EFNB2 in thymocytes development and function. Therefore, in the third article, we intended to investigate further the influence of EphB4, the preferred receptor for EFNB2, on T cell development. To achieve this goal, we specifically deleted EphB4 in mTECs by Keratin-5 (K5) promoter-driven cre recombinase, as EphB4 was expressed mainly in the TEC but not thymocytes. Our data showed that deletion of EphB4 in $K5^+$ mTECs did not influence T cell development and function.

In the thymus, the medulla contains two major subsets of epithelial cells which are $K5^+$ and $K8^+$ respectively.[154] Therefore, K5-cre is only active in a part of mTECs. To clearly reveal the function of TEC EphB4 in thymocytes development, it will be necessary to delete EphB4 expression in all TEC using both the K5 and K8 promoters to drive the cre expression. Also, giving the heavy redundancy of EFNBs and EphBs in the thymus, possible compensation of a loss of EphB4 in TEC by other EphB members could be expected. Therefore, it is highly possible that only when multiple EphBs in all TEC are deleted, would some phenotype in thymocyte development be revealed.

Although the importance of EFNB1 and EFNB2 in the immune system has been revealed according to findings from our lab and other researchers, the detailed mechanism of such function remains to be further investigated. [2, 88] EFNBs are capable of triggering both forward and reverse signalling upon binding to corresponding EphBs. In the thymus, EFNBs are expressed by thymocytes as well as TECs. [89, 91, 92] Therefore, thymocytes might receive forward and reverse signalling simultaneously. Hence, it will be of high interest to clarify which signalling of which direction plays a role in thymocyte development and functions. As a matter of fact, in our recent publication, EFNB1 and EFNB2 dKO bone marrow cells failed to reconstitute the T cell population as the co-transplanted competing WT SJL bone marrows cells. The SJL competitor expresses both intact Ephs and EFNs which could trigger forward signalling in the neighbouring dKO cells. Moreover, T cells lacking EFNB1 and EFNB2

demonstrated inferior immune responses. [40] Therefore, EFNB reverse signalling seems to be critical for T cell development and function.

EFNBs transduce reverse signalling via adaptor proteins, such as GRIP1, Grb4, Dsh, as well as PDZ-RGS3. [2] PDZ-RGS3 binds to PTB of EFNB1 and EFNB2 constitutively. It has been reported that PDZ-RGS3 contributes to negative regulation of CXCR4, a GPCR which is the ligand of CXCL12, and mediates cell migration in cerebellar granule cells when EFNB1 is activated. [28] In the immune system, CXCR4 signalling is also critical for thymocyte migration in the thymus as well as for T lymphocyte chemotaxis in tissues. [279] Moreover, CXCR4 signalling, in co-operation with Notch and the preTCR signaling, support T cell development beyond β-selection. [145] The expression of CXCL12 is largely homogenous in the cortex. This suggests that CXCL12 signaling alone may not be enough to polarize thymocytes and drive them all the way across the cortex to the capsule. [138] Under such circumstances, EFNB1 or EFNB2 may exert a fine-tuning function on CXCR4-mediated signalling during thymocytes migration and maturation in the thymus. Therefore, the spatial-temporal expression pattern of EFNB1/B2 as well as the presence of binding Ephs may function to determine the fate of developing thymocytes.

Although so-far, there is no knowledge regarding of the function of the other adaptor proteins in T cell development or responses, we cannot exclude their possible involvement.

In addition to signalling via the adaptor proteins, we reported a novel interaction between EFNBs and IL-7R in article 4, which retards IL-7R internalization. [39] Upon binding to IL-7, IL-7R undergoes internalization. [280] Our data shows that in dKO T cells, IL-7R undergoes faster internalization and transduces weaker signalling than that of WT upon IL-7 treatment. Both EFNB1 and EFNB2 are capable of stabilizing IL-7R on the cell surface during IL-7 engagement. [39] IL-7 is secreted by TECs in the thymus. During thymocytes development, IL-7Rα is first expressed at the DN stage. It is absent at the DP stage and re-appears at the SP stage. [281] IL-7 signalling is known to be essential for T cell development, homeostatic expansion, and differentiation into effector T cells. [282-284] It is possible that, when Lck-cre starts to be active at the DN3 stage, the diminishing EFNB1 or EFNB2 expression accelerates the internalization of IL-7Rα, and hence, dampens the survival and development of thymocytes from that stage one. Thus, combining the defects in T cell compartment we reported in the dKO mice in another paper, it is reasonable to speculate that EFNB1 and EFNB2 may also contribute to the fine-tuning of IL-7Rα signalling by influencing their internalization. Similar influence by EFNB1 and EFNB2 was also observed on IL-6R signalling. [40]

To summarize, with more and more evidences emerging, it becomes apparent that EFNBs may be involved in various aspects of the immune system from thymocyte development to immune responses. Based on current observations by us and other researchers, we believe that EFNB may function as a regulator which exerts fine-tuning roles for other major signalling pathways in the immune cells. (fig.1)

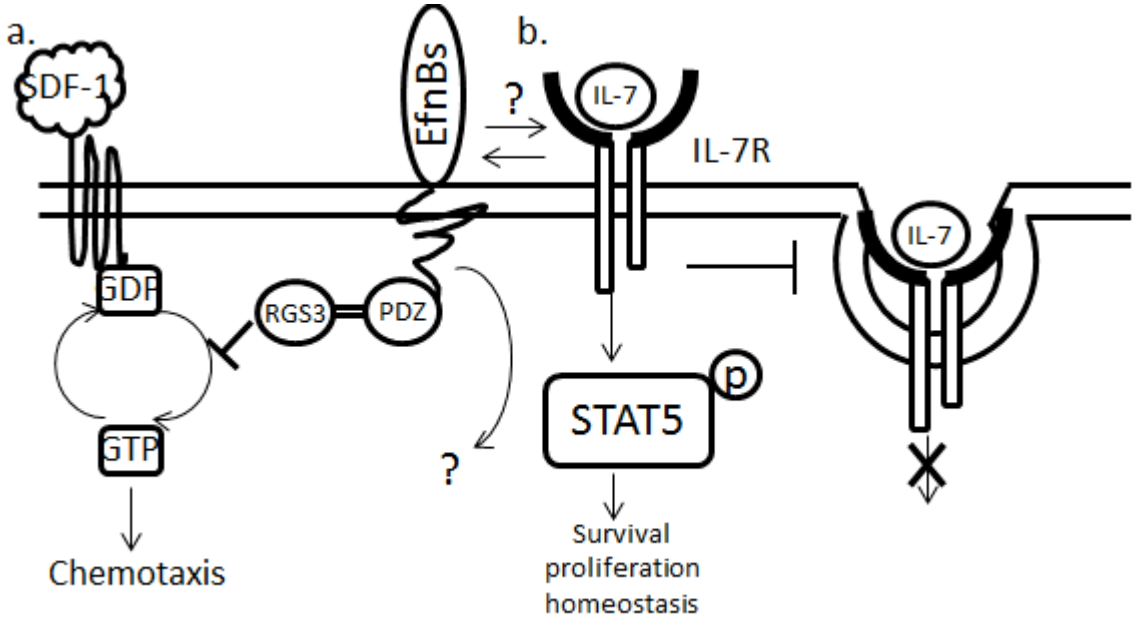


fig.3.1 possible involvement if EFNB in immune system. a) PDZ-RGS3 constitutively bind with PDZ binding motif of EFNB1 and EFNB2. Upon activation, EFNB1/B2 negatively regulate CXCR4 mediated chemotaxis. b) IL-7R undergoes rapid internalization upon IL-7 binding. EFNB1 and EFNB2 physically bind with IL-7R and stabilize it on cell surface, hence maintaining IL-7R downstream signalling.

III.2 Summary and future directions

In the present study, we have explored the function of EFNB1, EFNB2 as well as EphB4 in the immune system using conditional knockout strategy in mice. Our study has revealed the involvement of EFNBs in thymocytes development. To sum up, we have revealed that:

1) In the absence of EFNB1 or EFNB2, the thymus and spleen showed mostly normal subpopulations of T cell origin, only with relative increase in DN cell population in the thymus.
2) In the absence of EFNB1 or EFNB2, mature T cells had no apparent defects in their activation and proliferation or in their ability to differentiate into functional Th1, Th2, Th17 and Treg cells.

3) EFNB1 and EFNB2 physically interact with IL-7Rα

4) Efnb1 and Efnb2 retard IL-7Rα internalization in T cells, therefore, stabilizing IL-7R downstream signaling

5) EphB4 is expressed in thymus epithelial

6) Deletion of EphB4 in thymic epithelial has no impact on T cell development and function.

Given that the Lck promoter used in transgenic Cre expression, which leads to conditional deletion of the targeted genes, only starts to be active from the late DN2 stage, deletion of EFNBs in an earlier DN stage is needed to demonstrate the function of EFNBs in early thymocytes development. To further explore the mechanism of the involvement of EFNB in thymocytes development, it will be useful to employ an in vitro thymocyte culture model. In our ongoing research, we have being trying to manipulate the dKO thymocytes by lenti-virus transduced gene expression combining with a OP9-DL1 co-culture system in vitro. OP9-DL1 is a stroma-derived cell line which is capable of supporting DN thymocytes development to the DP stage. [285] We have constructed lenti-viruses which carry different deletion mutations of cytoplasmic domains of EFNB1 and EFNB2. By introducing the mutated forms of EFNB1 and EFNB2 into dKO cells, we can dissect and identify critical stretches of intracellular tails of EFNB1 and EFNB2 with regard to thymocyte development. We are also trying to knockdown several adaptor proteins which bind to EFNBs using lentivirus-mediated shRNAs expression. We expect that with information obtained from these two angles, we will have a clear picture of the pathway through which EFNBs exerts their function in thymocytes development. However, due to unknown reason, we cannot recover the developmental defect of dKO cells on OP9-DL1 system despite the high expression of EFNB1 or EFNB2. Thus, a more valid in vitro model is needed to continue the study.

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