Functional Study of Ephrins and Eph Receptors in the Immune System

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SUMMARY

Ephrins (EFNs) are cell surface ligands of Ephs, the largest family of cell-surface receptor tyrosine kinases. The function of EFNs in the immune system has not been well studied, although some EFNs and Ephs are expressed at high levels on certain leukocytes. The data presented here indicate that the EFNB subclass (EFNBs) and their receptors (EFNBRs) are expressed in peripheral lymphocytes and monocytes/macrophages, with T cells being the dominant $EFNBs^+$ and $EFNBRs^+$ cell type. Solid phase EFNBs-Fc in the presence of suboptimal anti-CD3 crosslinking enhanced T-cell responses in terms of proliferation, activation marker expression, IFN-γ production, and cytotoxic T-cell activity. After crosslinking, T cell receptor and EFNBRs congregated into aggregated rafts, and this provided a morphological basis for interaction between TCR and EFNBR signaling pathways. Further downstream, p38 and p44/42 MAPK was activated by EFNBR crosslinking. EFNBs, especially B1, and their receptors are also expressed in CD4CD8 double positive and CD8 single positive cells, and to a lesser extent, in CD4 single positive cells. Solid phase EFNB1 effectively protected thymocyte from CD3-induced apoptosis, while soluble EFNB1 significantly augmented such apoptosis. The effect of EFNB1 on thymocyte survival was more significant on populations expressing higher levels of EFNB1Rs. These results demonstrate that EFNBs and EFNBRs play pivotal roles in modulating T cell function and thymocyte development.

Key words: Eph; EFN; T cell; Thymocyte; costimulation; T-cell development.

Résumé

Les Ephrins (EFNs) sont un ligand membranaire de Ephs, la grande famille des récepteurs tyrosine kinase. La fonction des EFNs n'est pas encore bien définie dans le système immunitaire, mais certains EFNs et Ephs sont surexprimés dans les leucocytes. Les résultats présentés ici indiquent que la sous-famille des EFNBs et leurs récepteurs sont éxprimés dans les lymphocytes périphériques, les monocytes et les macrophages; dans les cellules T, les EFNBs et EFNBRs dominent. En phase solide, les EFNBs-Fc, en présence de concentrations suboptimales d' anti-CD3 amplifient la prolifération, l'activation des marqueurs d'expression, la production des IFN-g et l'activité cytotoxique des cellules T. Après inter-réaction (crosslinking), les cellules T réceptrices et EFNBRs se rassemblent en radeaux lipidiques. Ces derniers constituent une base morphologique pour l'interaction entre le TCR et l' EFNBR menant à l'activation de leur voie de signalisation. En aval, les p38 et p44/42 MAPK sont activés par l'inter-réaction de EFNBR. Les EFNBs, particulièrement EFNB1, et leurs récepteurs sont également exprimés par les CD4CD8 double positifs, les CD8 positifs et, moins fortenment par les CD4 positifs. En phase solide, EFNB1 protège efficacement les thymocytes suite à l'apoptose induite par CD3, même si EFNB1 en phase soluble augmente considérablement l'induction de l'apoptose. L'effet de EFNB1 sur la survie des thymocytes est plus significative sur les populations surexprimant les EFNB1Rs. Ces résultats démontrent que les EFNBs et EFNBRs jouent un rôle primordial dans la modulation de la fonction des cellules T et aussi dans le developpement des thymocytes.

Mots clé: Eph; EFN; T cell; Thymocyte; costimulation; T-cell development.

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LIST OF ABBREVIATIONS

AP-1	Transcription factor activator protein 1
APC	Antigen-presenting cells
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen-4
DP	CD4 CD8 double positive
ERK	Extracellular signal-regulated protein kinase.
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FasL	Fas ligand
FTOC	Fetal thymus organ culture
GEF	Guanine exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
Grb	Growth factor receptor bound protein
HVEM	Herpes virus entry mediator
ICOS	Inducible costimulatory molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAMs	Immunoregulatory tyrosine activation motifs
JNK	Jun N-terminal stress kinase Jun N-terminal stress kinase
KO	Knockout
LAT	Linker for activation in T cells
LIGHT	Homologous to Lymphotoxins, Inducible expression, competes with HSV Glycoprotein D for HVEM, a receptor expressed on T-lymphocytes
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIRR	Multichain immune recognition receptor

MLR	Mixed lymphocyte reaction
NF-AT	Nuclear factor of activated T cells
NF-κB	Transcription factor nuclear factor-κB
PAK	p21-activated kinase
PD-1	Phosphodiesterase-1
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 1,2-myristate 1,3-acetate
RT-PCR	Reverse transcriptase PCR
RTKs	Receptor tyrosine kinases
SH2	Src-homology 2
SLAM	Signaling Lymphocytic Activation Molecule
TCR	T cell receptor
Th1/2	T helper cell 1 and 2
TN	CD3 CD4 CD8 triple negative
TNF	Tumor necrosis factor
TRADD	TNFR-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF related activation-induced cytokine

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DEDICATION

I would like to dedicate this to my parents, Leihai Yu and Shaojie Peng, and to my daughter for their sufferings, and their constant encouragement and supports.

I would also like to dedicate this to my relatives and all of my friends here and in China, without them I really don't know how hard my time would be for …

I. INTRODUCTION

INTRODUCTION

In multicellular organisms, cells can interact with each other to achieve their biological functions. The cell surface receptors and their cell surface ligands are pivotal structures for such cell-to-cell communication. Receptor tyrosine kinases (RTKs) are a large group of cell surface receptors that possess intrinsic cytoplasmic tyrosine kinase activity. Functionally, they play critical roles in cell proliferation, differentiation, migration and metabolism.

There are several RTK families, such as the insulin, Eph, and the receptors for many growth factors, including the epidermal growth factor, platelet-derived growth factor, fibroblast growth factor and vascular endothelial growth factor. Ephs are the largest family of RTK, with a total of 15 members belonging to two subclasses (EphAs and EphBs subfamilies). They are membrane-bound glycoproteins and are activated by the binding of their cognate ligands, ephrins (EFNs). EFNs consist of 9 members of two subclasses, EFNAs and EFNBs. They are also membrane-bound. Both Ephs and EFNs can transduce the extracellular signal to the cytoplasm, either by phosphorylating tyrosine residues on the receptors themselves (autophosphorylation) and/or on downstream signaling proteins, playing their roles in regulating various cellular activities.

In comparison to the extensive studies done in the central nervous system, far fewer efforts have been made in understanding the function of Ephs and EFNs in the immune system. Our work aims to enhance our knowledge on the function of EFNs and Ephs in immune regulation.

I.1. Eph receptors and EFNs ligands

The term Eph originates from the name of a cell line, erythropoietin-producing human hepatocellular carcinoma, from which the first member of the family was identified (1). The ligands of Ephs, ephrins, may derive from two origins. One is the Greek word ejoros (or ephoros), meaning overseer or controller; the other is the abbreviation of Eph family receptor interacting proteins (2).

Since the first Eph member, EphA1, was cloned (1), new members of this family and their ligands were rapidly discovered. Many different names have been given to a same member, which is inconvenient for reference. In September 1996, a uniform nomenclature was recommended at the "Molecular Biology of Axon Guidance" workshop, and since then the new name of these largest RTK members have been widely adopted. A summarized nomenclature has been posted at the following website: http://cbweb.med.harvard.edu/eph-nomenclature. To date, 9 members of EphAs (EphA1-9) and 6 members of EphBs (EphB1-6) in the Eph family, and 6 members of EFNAs (EFNA1-6) and 3 EFNBs (EFNB1-3) in the EFN family have been identified (3, 4). Such a classification (A and B subfamilies) is based on sequence similarity and partner binding affinities. The members within each subclass share different degrees of homology. For example, the extracellular regions of EFNB1 show 50% amino acid identity with EFNB2, and 42% identity with EFNB3 (5). Detailed sequence homology dendrograms of Eph receptors and EFN ligands can be viewed at the website: *http://cbweb.med.harvard.edu/eph-nomenclature.*

I.1.1. Expression of EFNBs and Eph receptors

The expression of Eph and EFN protein has been shown in various tissues of zebrafish, xenopus, chicken embryos mice, rats and human (4). Physiologically, they are widely expressed during development, being most abundant in the nervous system and, to a lesser extent, in vascular endothelium and some specialized epithelia. They are also detected in some tumor cells or tumor-related tissues. The expression of Ephs and EFNs in various organs, including immune cells, is summarized in Table 1.

Table 1. Expression of Eph and EFN members

Name	Expression in organs or tissues
EphA1	In the embryonic or adult mouse: the liver, lung, kidney, thymus, and placenta (6, 7). In adult rat: the liver, lung, kidney, testis (8), and thymus (9). In human: high in the bladder, and small intestine; moderate in the lung, liver, colon, kidney, thymus, prostate and testis; low in the brain, spleen, and uterus (10).
EphA2	In the embryonic mouse: at the early stage, neurogenic crest cells of the facial, acoustic, and distal regions of limb bud mesenchyme; at later stage, the cartilaginous model of the skeleton, tooth primordia, infundibular component of the pituitary, various fetal tissue epithelia (11, 12, 13); high levels in the developing lung, kidney, intestine, and salivary gland (14). In adult mouse: the lung (11) and thymus (7). In the adult rat: high level in the lung, skin, small intestine, and ovary; low in kidney, brain, spleen, submaxillary gland (15), and thymus (9). In human: high level in the bladder, lung and kidney; moderate in the liver, spleen, small intestine, uterus and thymus; low in the brain, prostate and testis (10).
EphA3	In the embryonic mouse: the forebrain, midbrain, hindbrain, neural tube, branchial arches (1st & 2nd), somites, and limb buds (16). In the adult mouse and rat: the brain (17, 18, 19) and thymus or thymocyte (7, 9). In human: high level in the uterus, bladder and prostate; moderate in the brain, lung and testis; low in the thymus, liver, spleen, small intestine and kidney (10).

EphA4	In the embryonic mouse: multiple sites of the developing central nervous system, the striatum, ventricular zone of the cerebral cortex, hippocampus, thalamus, cerebellum, facial nucleus, and anterior horn of the spinal cord. In the adult mouse: the hippocampus, cerebral cortex, striatum, thalamus, granule layer of the cerebellum (14), fetal and adult B-lineage cells (20), and thymus (7). In rat: the adult brain (17) and thymus (9). In human: high level in the brain and testis; moderate in kidney; low in the thymus, heart, lung, spleen, pancreas, small intestine, muscle, placenta, bladder, uterus, prostate and bone marrow (10, 21).
EphA5	In the embryonic mouse: the telencephalon, olfactory bulb, and retina. In fetal and adult mice: high expression at the hippocampus (22). In the adult rat: the hippocampus, ascending central cholinergic nuclei and monoaminergic nuclei (23, 24).
EphA6	In the neonatal mouse: the neurons of the cochlear/spiral ganglion in the inner ear and whole brain (25). In the adult mouse: the thymocyte (7). In the embryonic rat: all over the body and head. In the postnatal and adult rat: only in the brain (23). In human: high in the testis; moderate in the brain and colon; low in the kidney, thymus, heart, lung, pancreas, small intestine, bladder, uterus, prostate and bone marrow (10).
EphA7	In the embryonic and postnatal mouse: predominantly in brain at embryo stage, also in various organs outside the nervous system during development: kidney, lung, limb buds, and to a lesser degree in the heart, liver, gut, and genital tubercle (26, 27). In the adult mouse: the brain, testes and, at lesser intensity, spleen (26), and thymus (7). In rat: strong expression from fetus to adulthood, especially in the brain, and may also in the retina and heart (28, 27, 29). In human: the brain, heart, kidney, and lung (20); high in the kidney, bladder, brain and testis; moderate in the spleen, small intestine, uterus and prostate; low in the lung, thymus and bone marrow (10).
EphA8	In the adult rat: the brain (30) and thymus (7). In human: high in the testis and moderate in the brain (10).
EphB1	In the embryonic mouse: the brain, cerebellum, and spinal cord during development; In the adult mouse: the hippocampus and cerebellum (14). In the embryonic rat: high levels in the cerebellum, brainstem, diencephalon, and caudate nucleus (31). In human: high in the brain and testis; moderate in the colon and kidney; low in the thymus, heart, lung, pancreas, small intestine, spleen, bladder, uterus and prostate (10).

EphB2	In the fetal mouse: the neuroectoderm (32), ventral midbrain, ventroposterior forebrain, ventral neural tube, the developing dorsal root ganglia, hindbrain, retina, posterior half of the foregut pocket, mesoderm around the heart (13), mesenchyme and heart (33). In the fetal rat: high in the brain cortex, modest in all fields of the hippocampus, and in a subset of Purkinje cells of the cerebellum, weakly in Schwann cells (17). In human: the lung, thyroid, placenta and muscle (21, 30); high in the colon and small intestine; low in the brain, thymus, heart, lung, spleen, pancreas, kidney, bladder, uterus, testis, prostate (10, 21).
EphB3	In the embryonic mouse: cerebellum, Purkinje cells, hippocampus (17), head mesenchyme, developing somites, foregut, first and second branchial arches, lateral mesoderm of the forelimb bud (13), venous endothelial cells, and some arteries (33). In the adult mouse: the brain, liver, lung, kidney, intestine, testes, placenta, muscle, and heart (34). In human: high in the colon, brain and lung; moderate in the uterus, prostate, small intestine, kidney and bladder; low in the thymus, spleen, testis and bone marrow (10).
EphB4	In the embryonic mouse: the epithelial lining of the brain and ventricles, thymus, lungs, heart, spleen, the entire glomeruli of kidney (34), and all major veins (33). In the adult mouse: brain, heart, lung, kidney, placenta, testes and muscle (35) In human: the fetal heart, lung, liver, and kidney, lesser signal in brain; adult placenta and pancreas (36); high in the lung; moderate in the spleen, liver, testis, uterus, kidney, bladder, colon (10); low in the skeletal muscle, heart (36), prostate, small intestine, thymus, and bone marrow (10).
EphB5	No documented data in rodent and human tissue. In the chicken: the thymus (37).
EphB6	In the adult mouse: high levels in the thymus and brain (38). In human: high levels in the thymus, moderate in the brain and testis, low in the lung, spleen, uterus, prostate, small intestine, kidney, bladder, colon and bone marrow (10).

EFNA1	In the fetal mouse: the lung, the epithelium of the gut, the ossifying bones of the face, salivary gland, central nervous system and low level in the connective tissue of septae in the thymus (39); the primitive streak, lateral mesoderm, tailbud, and at lower levels in the blood vessels (40); In the adult: the thymocyte (7). In the embryonic rat: the epithelial cells and the endothelial cells in lung, kidney, intestine, skin, skeletal system, teeth, heart (endocardium), and dorsal root ganglia neurons (41). In the postnatal rat: high levels in the lung, low levels scattered throughout the thymus (39). In human: high in the lung, colon and liver; moderate in the bladder, kidney, prostate and uterus; low in the brain, thymus, spleen, small intestine and testis (10).
EFNA2	In the embryonic mouse: high levels in the midbrain and lower levels in the dorsal anterior hindbrain; outside the nervous system, in the 1st and 2nd branchial arches, somites, and limb buds (16, 40). In postnatal mouse: the hippocampus in a lateral to medial gradient, and an increasing gradient from dorsal to ventral septum (22); the thymocytes (7). In human: high in the colon; moderate in the bladder, small intestine and liver; low in the brain, kidney and testis (10).
EFNA3	In embryonic mouse and rat: almost exclusively in the central nervous system besides the skin (42). In the adult mouse: thymocyte (7). In human: high in the brain; moderate in the colon; low in the thymus, lung, liver, bladder, kidney, prostate, uterus, small intestine and testis (10).
EFNA4	In the early embryonic mouse: the ectoderm of branchial arches, the forelimb bud, the anterior spinal cord, and throughout each somite the limb over the proximal limb and hand/foot plate (40); In the adult: thymocytes (7). In the embryo rat: the forebrain, olfactory bulb, hippocampus and cortex; In the adult: low level in the brain (31). In the human fetus: the heart, lung, and kidney of fetus; In the adult: high in the colon; moderate in the lung, kidney, bladder, small intestine and prostate; low in the thymus, uterus, liver, spleen, testis and bone marrow (10).
EFNA5	In mouse: the brain, heart, intestine, kidney, and at lower levels in the lung, liver (43) and thymocyte (7). In human: the heart and placenta (44); high in brain and kidney; moderate in the lung, spleen, bladder, colon and testis; low in the thymus, liver, prostate, uterus and small intestine (10).

EFNB1	In the embryonic mouse and rat: high level in brain, sciatic nerve (42) and kidney; moderate in the heart, lung, skeletal muscle and thymus; low in the liver (42, 45). In the adult human: high in the bladder, lung and colon; moderate in the kidney, spleen, small intestine, uterus and testis; low in the brain, liver, prostate and thymus (10).
EFNB2	In the fetal mouse: the hindbrain, branchial arches, in two intense regions of new somite formation, and a weaker signal in the forebrain (46). In the fetal human: the heart, lung, kidney and brain. In the adult human: high level in lung, kidney (47, 48) and colon (10); moderate in the brain, kidney, small intestine, testis and bladder; low in the thymus, spleen, liver, prostate and uterus (10).
EFNB3	In the mouse embryo: high level in the brain, lower levels in the heart, lung, and kidney. In the adult mouse: high levels in various sites of the central nervous system, and also in the heart (49). In the adult human: high in the brain; moderate in the uterus; low in the thymus, liver, kidney, small intestine, testis, bladder, prostate, lung and colon (10).

The expression of some Eph and EFN members in immunological compartments has been reported during the past years. Hek (EphA3) protein was detected in a pre-B cell acute lymphoblastic leukemia cell line (LK63) in early 1992 (50), and later on, in chicken embryo thymus (37). Following that, several other Eph and EFN members were found in mice, rat and human lymphoid cells or immune organs (6, 7, 9, 14, 15, 20, 26, 38, 42, 45, 47, 48, 51, 52, 53). However, the majority of these informations were derived from the detection at the mRNA level, and some contradictory results were reported. For example, EFNA1, A3 and A5 were undetectable in thymocytes in early studies (54), but were later found to be expressed in these cells (7). Such a discrepancy may be due to the sensitivity of the different

assays employed. In the last three years, more and more Eph members and their ligands have been detected in the immune system (7, 9, 10). A general expression profile of Eph and EFN members in major immunological compartments is summarized in Table 2.

Table 2. The expression of Eph and EFN members in murine or human immune system

Eph/ EFN	Spleen	thymus	Reference
EphA1	+	++	6, 9, 10
EphA2	+ / ++	+ /++	15, 9, 10
EphA3	+	+	7, 9, 10
EphA4	+	+	21, 9, 10
EphA5			
EphA6		+	10
EphA7	++	+	26, 10
EphA8		+	7
EphA9			
EFNA1	+	+	39, 10
EFNA2		+	10
EFNA3	+	+	7
EFNA4	+	+	7
EFNA5	++	+	7, 10
EFNA6			
EphB1		+	10
EphB2	+	+	30, 21, 10
EphB3	+	+	10
EphB4	+	++	34, 36, 10
EphB5			
EphB6	+	+++	38, 10
EFNB1	+ / ++	+	42, 55, 10
EFNB2	+ / ++	+	47, 48, 10
EFNB3	+	+	10

There are variations in the distribution and expression levels in different species, tissues and developing stages. Patterns shown are for embryo or adult of mice, rat or human. The +++, ++, + represent strong, moderate, weak expression.

I.1.2. The structure of and interaction between EFNs and Eph receptors

The Eph receptors are type I transmembrane proteins (56) (Fig. 1 A). The extracellular sequence has an N-terminal ligand-binding domain, a cysteine-rich domain and two fibronectin type III domains. In the cytoplasmic sequence, almost all the Eph members have a juxtamembrane segment, a highly conserved tyrosine kinase domain (57, 58), a sterile α motif (SAM), and a C-terminal PDZ-binding motif. The cysteine-rich region close to the ligand-binding domain is believed to be involved in receptor–receptor interactions (59). The biological function of the two fibronectin repeats is not clear so far, but they possibly interact with other proteins (60). The SAM domain may have a role in receptor dimerization (61) or oligomerization (62), and it may also interact with adapter proteins (4).

All EFNs share a basic structural component, i.e., a unique conserved extracellular receptor-binding domain at the N-terminus. In the EFNB subclass, an additional highly conserved cytoplasmic domain and a C-terminal PDZ-binding motif are also found, and these are believed to be involved in protein-protein interactions that mediate membrane localization and reverse signaling (63). The EFNA subclass members have no transmembrane region, and they are linked to the cell by a glycosylphosphatidylinositol (GPI) linker (Fig. 1 B).

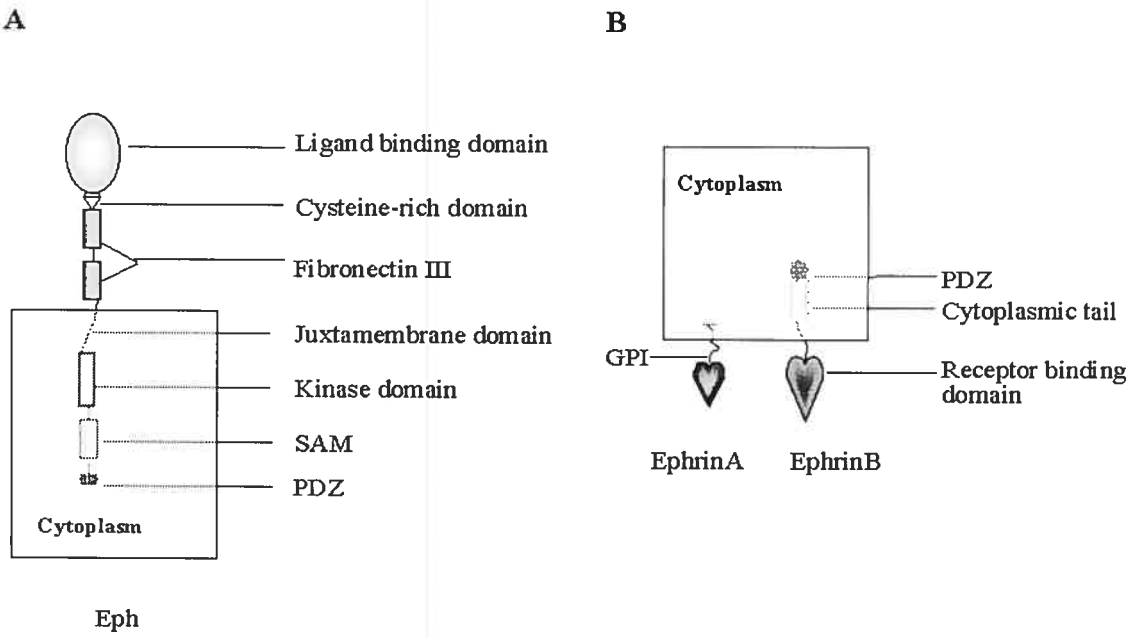

Fig.1. The structure of Eph and EFN

A. The Structure of Eph. B. The Structure of ephrinA and ephrinB. Abbreviations: GPI, glycosylphosphatidylinositol; SAM, sterile-α-motif; PDZ, PSD95/Dlg/ZO1-binding motif.

The consequence of EFN and Eph interaction is the initiation of the signaling events in the receptor bearing cells (forward signaling), or signaling into the ligand bearing cells (reverse signaling). It is also possible that the forward and reverse signaling, occur at the same time (bidirectional signaling).

The Eph-EFN-mediated signaling depends on the association of Eph receptors and ligands. When Eph and EFN meet, there is initial conformational change in both the receptors and ligands to achieve a better fit for the receptor-ligand interaction. Receptors and ligand oligomerization, i.e., trimers composed of two receptors and one ligand, or tetramer of two

receptors and two ligands, is basically needed to trigger forward and /or reverse signaling (64).

The interaction between Eph and EFN is promiscuous, and recent evidences underline this characteristic. In general, as illustrated in Fig.2, the EphAs interact with EFNAs, and the EphBs interact with EFNBs. However, some exceptions exist. For example, in addition to binding to all of the five EFNA subclass members, EphA4 can also bind to EFNB2 (49, 65). Most recently, EphB2 and EFNA5 were shown to be able to bind one another with high affinity (66). This further complicates the existing profile of receptor-ligand interaction between different Eph and EFN members.

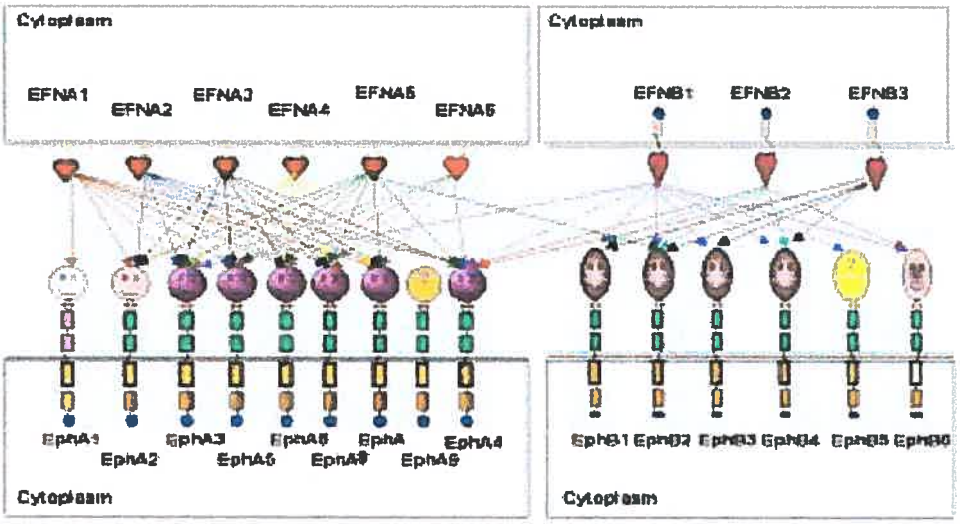

Fig.2. Interactions between Ephs and EFNs

Solid lines represent the proven interactions between the receptors and ligands. Dashed lines indicate possible interactions. (Data come from: 65, 5, 54, 67, 68, 7, 69, 70, 66)

In addition to the promiscuity, another characteristic of the Eph ligand-receptor interaction is that ligands and receptors need to be membrane-bound to be effective in triggering forward or reverse signaling. The soluble forms are not only inactive, but can be antagonistic to the membrane-bound ligands or receptors (42, 44, 71). Such a characteristic constitutes the basis for the requirement of direct contact between Eph and EFN-bearing cells to initiate the biological function of Eph and /or EFN.

I.1.3. Function of EFN and Eph receptors

Eph and EFN have been implicated in the regulation of many critical events during development, and to some extent in adult life.

So far, the best-defined biological function of Eph and EFNs is the mutual "repulsive" or "attractive" effects on cells expressing Eph and EFN, such effects resulting in structural modulation in tissue morphology, such as axon guidance (72), vascular development (73), and cell migration (74).

The roles that Eph and EFN may play in adulthood are related to angiogenesis (75) , carcinogenesis (76), tumor metastasis (77), and T cell regulation (78, 53, 9, 7, 79, 80).

I.1.3.1 Eph/EFN in the development of the nervous system

One of the most important functions of Eph-EFN interaction is their involvement in axon guidance or pathfinding. Both Ephs and EFNs are expressed on axons and cells of neural

origin and their expression may follow a specific space and time pattern. During development or structural reformation of the nervous system, the Eph and EFN bearing cells receive signals from each other and thus modify their growth pattern. Although the effect of Eph/EFN interaction may also be pro-adhesive (81), the majority of evidence showed that such a signal is repulsive. For example, EphA3 expression follows gradient in the tectum and retina. When EFNA2, a ligand of EphA3, is overexpressed in the complementary region of Eph expression, the axons turn to an abnormal direction (82, 83, 84, 85). Such a repulsive function is also supported by observations from mice with mutations of some Eph members (86, 87, 88).

The importance of Eph receptors and EFNs in the adult has drawn attention in recent years. Significant expression of some Eph and EFN members has been found in the adult nervous system (10), and a role for EphB in the regulation of neural stem cell migration has been described (4). How important Eph-EFN interaction is in the regulation of the adult nervous system needs to be further elucidated.

I.1.3.2 Eph/EFN in angiogenesis

In addition to their function in the nervous system, Eph receptors and EFN ligands are also involved in other morphogenic events, such as angiogenesis. Several Eph and EFN members play a critical role in normal vascular formation. For example, EFNA1 and EphA2 are highly angiogenic (89), although the effect is not solely caused by the Eph-EFN interaction, and may depend on the contribution from other molecules (90). Some EphB and EFNB subclass

members, such as EphB4 and EFNB2, also participate in the development of the cardiovascular system (33, 91, 73, 92). The pivotal roles of Eph and EFN members in angiogenesis have been demonstrated both in vitro and in vivo, and this topic is reviewed in detail by Brantley-Sieders et al. (93). In accordance with the observation of the formation of topographic projection maps in the nervous system (81), Eph/EFN interaction can generate both positive and negative biological effects on angiogenic cells, to promote neovascularization (74), regulate arteriovenous differentiation and control vascular homeostasis (94).

I.1.3.3 Eph/EFN in tumor tissue

Mutation and/or over-expression of receptor tyrosine kinases have been shown to be associated with tumorigenesis a decade ago (95). The Eph family has been known to be related to cancers since the first member, EphA1, was discovered (1). Several Eph family receptors are found in tumor tissues or tumor-derived cell lines. For example, EphA1 is over-expressed in various tumors, including breast, lung, liver, colon, and prostate carcinomas (1, 8, 96); some other EphA and B members, including EphA2, A3, A4, B2, B3 and B4, are also over-expressed in tumor cell lines (50, 97, 98, 35, 99, 100).

The Eph expression levels seem to be related to the malignancy of tumors. Some Eph members are detected in the undifferentiated and invasive mammary tumors, but not in well-differentiated and non-metastatic tumors (35). However, a comprehensive investigation on cells derived from normal tissues, and non-invasive and invasive tumors, respectively,

shows diverse Eph/EFN expression profiles. Upregulation of some Eph and EFN members are positively related to tumorigenesis and/or invasiveness, while some other members are downregulated (101).

Although over-expression of EphA1 in mouse NIH 3T3 fibroblast cells induces colony formation in soft agar and tumor formation in nude mice (102), few data are available on the causal effect of Eph/EFN on tumorigenesis. Recent data support a model in which Eph-EFN interaction promotes tumor growth by stimulating angiogenesis (103).

Thus, although a growing body of evidence supports the involvement of EFNs and Eph molecules in tumor angiogenesis, the data are not sufficient to prove a causal effect of Eph and EFN in inducing cell transformation. On the other hand, Eph and EFN are quite possibly involved in the metastatic phase of cancers and the angiogenesis of tumor tissues.

I.1.3.4 Eph/EFN in the immune system

The expression of some of the Eph kinases and their ligands on immune cells has been documented (Table 1 and 2). However, the function of this largest RTK family in immune responses was basically unknown until we started our project in 2001. We have reported that a kinase defective Eph family member, EphB6, is capable of transducing signals into T cells (104). Activation of EphB6 with solid phase anti-EphB6 mAb results in Jurkat cell apoptosis. Later on, we found that EphB6 can augment normal human T-cell response to Ag stimulation (53). However, probably due to overlapping functions of Eph kinases, no abnormal

phenotype in terms of thymocyte structure and subpopulation composition is found in EphB6$^{-/-}$ mice (105). It is to be noted that no detailed study on the functional defects in the EphB6$^{-/-}$ immune system was conducted in that study. In the same year, Munoz et al. reported that a few soluble recombinant EphAs and EFNAs interfere with T-cell development in thymic organ culture (9), and Sharfe et al. described that several EFNs, notably EFNA1, inhibit T-cell chemotaxis (78). Recently, Roifman's group (80) showed that EphB6 can influence T cell immune response by downregulating anti-CD3-induced IL-2 secretion and CD25 expression in a ligand-dependent manner. When murine thymocytes are stimulated with EFNB1, TCR-mediated apoptosis is inhibited, suggesting that EphB6 may be involved in thymocyte development during positive or negative selection.

In addition to the results mentioned above, in situ hybridization revealed strong EFNB1, B2 and B3 mRNA signals in the cortex of the thymus and white pulp in the spleen (106, 107, 108, 109). Taken together, the data raise the possibility that EFNBs might play pivotal roles in regulating T-cell development and function.

I.1.4. Signaling by EFN and Eph receptors

Although activation of Eph receptors may follow some general rules established for other RTKs, the signal transduction by the Eph receptors and EFN ligands has a number of unique features. The EFN ligands need to be membrane-bound to be effective, and misregulated tyrosine kinase activities of many Eph members are not oncogenic in contrast to other

receptor tyrosine kinases. It is also unusual that the Eph-EFN signaling is bi-directional, i.e., both receptors and ligands can transmit signals into cells.

I.1.4.1. Forward signaling by Eph receptors

The exact process of Eph-EFN interaction is not well understood for each receptor and ligand member. The crystal structure analysis on some Eph/EFN members revealed some clues in recent years (61, 110, 111, 112). Before ligand binding, the kinase domain in Eph sticks to the juxtamembrane segment, on an autoinhibited conformation. Upon ligand binding, juxtamembrane tyrosines of Eph can be phosphorylated; this releases the autoinhibition and free phosphotyrosine sites for SH2 domains-bearing signaling proteins (111). In addition to such a de-autoinhibition mechanism, some phosphorylation-independent interactions between EphB and substrate proteins also exist (113).

Many SH2-domain-bearing adapter proteins are involved in the Eph signaling. For example, Grb2, Grb7 and Grb10 bind to EphB1 (114, 115); Abl and Arg bind to EphB2 (113). Through their SH2 binding domains, the adapter proteins, such as Shc and Nck, can interact with Eph receptors and influence the activation of the JNK or ERK pathway (116, 117). The recruitment of Shc may result in tyrosine phosphorylation, which provides binding sites for the SH2 domains on other adapter proteins. Nck can interact with downstream effectors, such as Pak3 and WASP (72, 118). Notably, Abl and Arg may interact with Eph through their C-terminal tails other than the SH2 domains (113).

Some small GTPases of the Rho family (Rho, Rac and Cdc42) are involved in Eph receptor

signaling (78, 119, 120). Examples come from both Eph A and B subfamilies, although there are some differences between these two groups. Stimulation of EphA receptors can activate Rho GTPases (119), and downregulate the activation of Rac (120) and Cdc42 (78). On the other hand, EphB can influence the intersectin-Cdc42-WASP-actin pathway (121), or induce translocation of Rho-GEF kalirin to the site where other target signaling molecules exist, and further activate downstream targets, such as small GTP binding protein Rac1 (ras-related C3 botulinum toxin substrate 1, rho family) (122).

Ligation of EphB receptors with EFNB ligands can also activate Rap1 (123, 124, 125), a R-Ras-like protein that positively modulates integrin-mediated adhesion (126, 127). Such activation may be mediated by some other adaptor proteins (128), such as Crk (123).

The Src non-receptor kinases may also be related to Eph signaling (129). Some Src proteins can be activated after Eph ligation, and the Src family kinase Fyn is important to Eph signaling in modification of cell adhesion (130, 131).

Recently evidences have shown that Eph/EFNs signaling interferes with some growth factors, e.g., vascular endothelial growth factor (VEGF) (90). The effect of EphA and EFNA on angiogenesis is achieved with the involvement of VEGF, and phosphoinositide 3-kinase (PI3K) and Rac1 GTPase are also related to such an activity (132). EphA ligation may inhibit proliferation of prostatic epithelial cells and endothelial cells through downregulation of the Ras/MAPK cascade (133, 134), which is activated by some growth factors, e.g., epidermal growth factor (EGF) and VEGF. However, such signaling events are not universal. For

instance, EFNA1 inhibits proliferation in prostatic epithelial cells and endothelial cells, but not fibroblasts (133).

Our laboratory is the first one who discovered that the Cbl is involved in Eph signaling in T cells (104, 135, 136, 137). EphB6 cross-linking by Ab in Jurkat cells causes Cbl dephosphorylation (104). Cbl phosphorylation is observed in both Jurkat cells and peripheral blood T cells stimulated by EFNA1 (79); such EphA activation-induced Cbl phosphorylation may employ adapter protein Crk-L and Crk-II, with an involvement of Src family kinases. However, stimulation of EphB receptors with EFNB1 does not induce Cbl phosphorylation in Jurkat cells (79). Recently, Roifman's group demonstrated that EFNA1 stimulation of Jurkat cells could cause down-regulation and degradation of endogenous EphA3 receptors. EphA receptor expression is down-regulated in cells overexpressing of wild-type Cbl, suggesting that Cbl might be a negative regulator of Eph receptors (80). In addition, they found that TCR-mediated activation of the JNK pathway is selectively inhibited upon stimulating overexpressed EphB6 with EFNB1. According to their speculation, small GTPase Rac1 is possibly involved in this event.

So far, although the repertoire of molecules involved in Eph forward signaling is expanding, the available information is insufficient to develop a complete architecture of these Eph signaling pathways.

I.1.4.2. Reverse signaling by EFN ligands

Although the existence of EFN reverse signaling has been observed for almost ten years (138), and the reverse signaling is one of the critical feature of Eph-EFN interaction (139, 140, 92), the mechanism of reverse signaling still lacks details.

Several evidences suggest that EFNB-induced reverse signaling is mediated by proteins bearing SH2 domains (141, 142, 143), and that Src family kinases (SFKs) is responsible for EFNB phosphorylation upon Eph receptor engagement (144, 145). In the EFNB-expressing cells, ligation with EphB causes rapid recruitment of SFKs, and induces transient SFK activation, which is possibly mediated by phosphorylation of the tyrosine residues and the engagement with PDZ domain proteins (145). Other PDZ-bearing proteins, such as RGS3, and SH2-bearing molecules, e.g., Grb4, can also induce the reverse signaling upon EFNBs-receptor ligation (142, 146, 63). RGS3 can further regulate downstream G protein signaling. Grb4 can link EFNBs to a number of signaling molecules, such as the Cbl-associated protein, the Abl-interacting protein-1, dynamin, PAK1, and axin (63). Activation of EFNB1 increases Fak activity, redistributes the Fak-binding protein paxillin, and leads to disassembling of focal adhesions (63, 123).

EFNA can also induce reverse signaling. Some evidences demonstrate that EFNA can recruit the Src family kinase Fyn to lipid rafts upon Eph ligation. As a consequence, tyrosine phosphorylation of a 120 kD lipid raft protein and activation of MAP kinases are observed

(147, 148); however, the mechanism is unclear.

I.2. T-cell development in the thymus

The thymus is the cradle of T cells, and is a complex primary lymphoid organ composed of stromal cells and immigrant hemopoietic cells. Anatomically, the thymus can be divided into three regions: the subcapsule region, the cortex, and the medulla (9). Hemopoietic stem cells enter the thymus and go through the early stage of development within the subcapsule and cortex where they lose the ability of differentiating to non-T cell lineages, and are gradually committed to the T-cell lineage (149).

At the earliest stage in the murine thymus, the lymphoid precursors are characterized as $CD4^{lo}CD8^{-}CD25^{-}CD44^{+}c\text{-}Kit^{+}$ (150). Between 12.5 and 14.5 of embryonic days (E12.5~E14.5), lymphoid precursors differentiate to the T-cell lineage; the thymic epithelial cells are not able to sufficiently support T-cell development at the stage earlier than E12.5 (151). Some cell surface markers, such as CD25, CD44, CD117 (c-kit) and CD127 are considered to be indicators of T-cell lineage commitment (152, 153), and the Notch signaling is believed to play a pivotal role to the commitment (154). At this stage, CD3 CD4 CD8 are negative in the T cell precursors; so these cells are often named TN cells. These TN can be further classified into TN1 ($CD44^{+}CD25^{-}CD117^{+}CD127^{-}$), TN2 ($CD44^{+}CD25^{+}CD117^{+}CD127^{+}$), and TN3 ($CD44^{lo}CD25^{+}CD117^{lo}CD127^{lo}$). The TN2 cells upregulate the expression of pre-TCR (pT) gene and begin to rearrange the TCR gene (152,

153). Then, along with further TCR gene rearrangement, the TN3 cells definitely commit to the T-cell lineage. These cells will turn to be $CD4^+CD8^+CD3^{lo/int}$, also called double positive (DP) cells (155).

The DP cells then undergo extensive positive and negative selections, and differentiate into mature CD4 or CD8 single positive T cells that express high levels of CD3-associtaed TCR, and are ready to move out of the thymus as functional and self tolerant mature T cells.

I.2.1. Positive selection

Positive selection refers to a process that choose the developing DP cells capable of recognizing self-MHC (major histocompatibility complex) molecules for further differentiation and survival (156, 157).

The positive selection requires the contact between TCR on the DP thymocytes and the MHC-peptide complex provided by other cells (158, 156, 159); the majority of MHC for efficient contact is supplied by thymic stromal cells at the subcapsule and cortex region (160). Both the concentration and specificity of antigenic peptides presented by MHC are important in determining the strength of the TCR signaling, and thus the fate of the DP cells (158, 161, 162, 163, 164, 165). Obviously, the molecules that can regulate TCR signaling, such as CD80 (166), Cbl (167) and LAT (168), may influence the outcome. Notably, it seems that the cortical epithelium expresses some unique molecules that may provide additional signals required during the positive selection (156, 163).

The thymocytes that pass through the positive selection will further develop into $CD4^+CD8^-$ or $CD4^-$ $CD8^+$ single-positive (SP) thymocytes. Those who recognize class I MHC develop into $CD8^+$ cytotoxic T lymphocytes (CTLs), and the others who interact with class II MHC commit to helper $CD4^+$ cells (169, 170, 171).

I.2.2. Negative selection

Negative selection is a process to delete the maturing DP cells capable of recognizing self MHC-peptide complex with high affinity. This is a critical mechanism to keep the mature T cells from being reactive to self-Ag.

Negative selection of thymocytes takes place either in the cortex at the cortex-medulla junction or within the thymic medulla (172, 173); it occurs probably not earlier than the $CD4^{low}CD8^{low}$ TCR^{int} $CD69^+$ stage (174). Dendritic cells (DC) play an important role for this process (175). Some accessory molecules, which can influence TCR signaling, such as CD40/CD40L and CD28, also play important roles in negative selection (174, 176). However, their involvement appears not just as simple as co-stimulation or modulation of TCR signaling. For example, Fas (174) and LIGHT (177) are able to affect negative selection, but FasL does not have obvious effect (174), suggesting the existence of a more complex mechanism than that employed in the mature T cells.

I.3 Co-stimulation in T-cell activation

The recognition of antigens by specific lymphocyte receptors constitutes a fundamental immune reaction, and T cells are a critical component for an effective immune response against a wide range of pathogens. Mounting an appropriate T-cell response depends on delicate regulation of lymphocyte activation. To become fully activated, two independent signals are required. The first is an antigen-specific signal that is delivered through theTCR. The second one is termed co-stimulation that works independently of TCR. Accumulating knowledge indicates that co-stimulation plays a crucial role in T-cell activation (178), proliferation (179), differentiation to effector (180) or memory status (181), anergy (182), and apoptosis (183).

The co-stimulation model (or two-signal theory) of T-cell activation was first proposed by Lafferty and colleagues (184). Many cell surface molecules and cytokines, such as B7 (185), CD44 (186) and IL2 (187) can transmit signals to enhance T-cell activation. Accumulated evidence indicates that bidirectional communication between T cells and accessory cells exists, and that this kind of interaction can evoke mutual stimulation (188). Now it becomes clear that interactions between responder lymphocytes and "accessory" cells represent a critical event in the T-cell activation process; such interaction is usually called co-stimulation. However, there is still no general agreement on what exactly defines "co-stimulation". The use of "negative co-stimulation" for inhibitory signaling events further confuses the terminology. For the convenience of the discussion here, a co-stimulatory molecule is defined as an initiator of a positive signal by increasing TCR avidity (for example, adhesion

molecules) or enhancing the recruitment of tyrosine kinases to the TCR complex (for example, the co-receptor CD4) (188). Those inducing negative signals are not called co-stimulatory molecules, but are nevertheless discussed.

I.3.1 Co-stimulatory molecules

The first cell surface molecule shown to function as a co-stimulatory molecule is CD28 (185). Since its identification, the number of candidate co-stimulatory molecules has significantly increased (189, 190).

Most molecules that induce co-stimulation can be divided into two classes based on sequence homology.

The first class is composed of members of the Ig superfamily, including CD28 family members. These molecules share several structural and functional features. All of the CD28-like receptors are type I transmembrane glycoproteins (191). The CD28 family comprises CD28, CTLA4, ICOS (H4, AILIM) and PD-1; CD28 appears to be the major co-stimulatory molecule for the activation of naive T cells. The ICOS mainly works on activated or effector T cells. The other two receptors, CTLA-4 and PD-1, although they share structural homology with CD28 and ICOS, appear to be both inhibitory in nature. The ligands of the CD28 family members belong to the B7 family, and these ligands are also members of the Ig superfamily.

The second class of co-stimulatory molecules comprises members of the TNF receptor (TNFR) family, including CD40, the major B cell co-stimulatory molecule, as well as OX-40, 4-1BB, CD27, CD30, and HVEM (herpes-virus entry mediator). The ligands for these receptors are membrane-bound members of the TNF family, e.g., OX40 ligand (OX40L), 4-1BBL, CD70, CD30L and LIGHT, respectively. The co-stimulatory TNFR-family members are mainly expressed on T cells, and their ligands, on antigen-presenting cells (192, 193).

I.3.1.1. The major co-stimulatory molecule CD28

CD28 is constitutively expressed on the surface of 80% of human T cells (all $CD4^+$ cells and about 50% of $CD8^+$ cells) and on virtually 100% of murine T cells (185, 194). Studies in vitro and in vivo have shown that CD28 is the primary co-stimulatory molecule for naive T cells, and $CD4^+$ cells are more dependent than $CD8^+$ cells on CD28 co-stimulation in vivo (195, 196).

The major function of CD28 signaling is to act in concert with TCR stimulation. CD28 ligation can enhance the transcription and stability of IL-2 mRNA, partially through cyclosporine insensitive signals (197). In recent years, accumulated data reveal more broad and proximal intersection of CD28 signals with those mediated by TCR (198, 199, 200). Ligation of CD28 on naive T cells by its ligand B7-1 or B7-2 on APC drastically enhances

the TCR signaling that activates protein tyrosine kinases (PTKs). The activated PTKs in turn phosphorylate critical scaffold proteins, such as the linker for activation of T cells (LAT) and the SH2 domain-bearing leukocyte-specific phosphoprotein of 76 kD (SLP-76) (201). Such events are critical for the further recruitment and activation of other key signaling molecules, such as phospholipase Cγ-1 (PLCγ1), growth factor receptor-bound protein 2 (Grb-2), son of sevenless (SOS), and protein kinase C theta (PKCθ) (202, 203, 204, 205, 206, 207). Through these molecules, the CD28 signaling triggers downstream pathways leading to the activation of the nuclear factor of activated T cells (NF-AT), activator protein-1 (AP-1), and nuclear factor kappa B (NF-κB) transcription factors (203, 208, 209, 210).

Activation of PI3K is one of the controversial issues in CD28 down stream signaling. PI3K is an intracellular signaling enzyme that regulates a wide range of cellular functions (211). In T-cell activation, PI3K may tyrosine phosphorylation–dependently associate with the cytoplasmic tail of CD28, phosphorylate membrane phospholipids phosphatidylinositol-4,5-bisphosphate (PIP2), and generate phosphatidylinositol-3,4,5-triphosphate (PIP3) that can activate a serial of downstream signaling targets. Recently, there are evidences either for and against an essential role of PI3K in CD28 signaling (212). By introducing mutant CD28 constructs, which cannot bind PI3K, into CD28-deficient mice, two groups of researchers obtained strikingly different results. One group (205) reported that CD28 Y189F transgenic T cells severely reduce the ability of proliferation and IL-2 secretion at the early stage of anti-CD3 and anti-CD28 Ab stimulation, although the later responses are intact. On the other

hand, the IL-2 production is totally lost when the cells are stimulated with PMA and anti-CD28 at any time point. In vivo test further demonstrated that CD28 Y189F transgenic spleen cells do not induce acute graft-vs-host reaction. Another group (213) showed that the CD28 mutant (Y170 in mouse) is more susceptible to radiation-induced death, but does not significantly compromise the ability of IL-2 production, cell proliferation and B cell help. The contradiction might be caused by different mutation points; this suggests that the performance of CD28 might be binding-site dependent. However, in another separate study, it was observed that T cells from mice lacking the p85 α subunit of PI3K appear to function normally (214). As the p85 α subunit is believed to be required for CD28 binding to PI3K, this observation suggests that PI3K is not required for CD28 signaling. Thus, the role of PI3K in the CD28 co-stimulation pathway remains undetermined.

Vav is another important signaling protein that regulates AP-1 activation, and plays an important role in the TCR/CD28-induced stimulation (204). Acting as a guanine-nucleotide exchange factor for Rac and Cdc42, Vav allows these molecules to switch from an inactive GDP-bound state to an active GTP-bound state (215). Signals from either CD3 or CD28 can independently phosphorylate Vav and activate it, with CD28 being more potent and inducing more sustained phosphorylation. Vav may provide a link between CD28 and Rac/Cdc42–controlled events, possibly through an adapter protein complex composed of SLP-76, LAT, and Grb2 (216). Vav may receive some CD28 signals via PI3K (217), and activate Rac-1, a ras-like guanosine triphosphatase (GTPase), and induce actin cytoskeletal rearrangement.

This cytoskeletal change is critical for activation-induced clustering of TCR and aggregation of lipid microdomains (rafts) that harbour a variety of signaling molecules (199, 200).

In addition to PI3K and Vav, Akt has received attention recently. Akt, also called protein kinase B or PKB, is a serine/threonine kinase that is recruited to the plasma membrane when its pleckstrin homology domain binds to the phospholipid products of PI3K. In a PI3K-dependent way, Akt can be phosphorylated by cross-linking CD28 independent of TCR ligation (218, 219). The activation of Akt is achieved after its phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) and other kinases at the membrane (220). The activated Akt further phosphorylates downstream targets. Notably, persistent activation of Akt can stimulate IL-2 production in mature CD28-deficient T cells (219), even though Akt has no effect on proliferation of CD28-deficient cells. It seems that Akt requires cooperative signaling from other molecules to mediate CD28 signaling. However, what molecules work downstream of Akt to induce the CD28-independent IL-2 production is so far unknown.

Cdc42 is another signaling molecule that is involved in the CD28-mediated signaling pathway. It plays a role in cytoskeletal rearrangement through the action of the Wiscott-Aldrich syndrome protein (WASP) and augments IL-2 synthesis via MAPK activation (215).

I.3.1.2 Other co-stimulatory molecules related to T-cell activation and differentiation

Although CD28 is a key co-stimulatory molecule, it does not account for all co-stimulatory

functions in T cells. The existence of CD28-independent pathways has been demonstrated by convincing evidences derived from CD28 null mice (195), and from APCs lacking B7.2 (220, 221). Accumulating evidence suggests that other co-stimulatory molecules can compensate for the absence of CD28 signaling; ICOS is such an alternative that has attracted much attention recently. The structure of ICOS is closely related to that of CD28, but there is no detectable binding of ICOS to B7.1 or B7.2. The ligand for ICOS, named ICOSL, is a novel B7 family member (190, 222). On naive T cells, ICOS is either absent or expressed at very low levels, while it is upregulated upon stimulation (223). Co-stimulation by ICOS contributes to the production of effector cytokines IFN-γ, TNF-α, IL-4, IL-5, and IL-10, but much less for IL-2.

Multiple molecules on T cells, such as CD2 (220), CD5 (221), CD9 (222) and CD44 (186), have the ability to costimulate T cells. In the presence of immobilized submitogenic doses of anti-CD3, mAbs against these co-stimulatory molecules all induce activation of naive T cells, and the proliferation rates determined 2 days after these co-stimulations are comparable to that induced by anti-CD28. Remarkably, co-stimulation by these molecules induces limited amounts of IL-2. Regarding the mechanism of such discrepancy, recent reports suggest that CD28 delivers a signal different from that derived from the others (227). There are several examples to support this notion:

1). Analysis of the CD28RE (interleukin-2 CD28 response element) of the IL-2 promoter shows specific transcription factor recruitment at the CD28RE element upon induction by

B7-1/SEE (227).

2). Although CD58 (LFA-3, ligand of CD2) co-stimulation is able to induce T-cell proliferation as well as IFN-γ and IL-4 production at similar levels as in cells induced by B7-1, IL-2 promoter activity and production of IL-2 are only seen after B7-1 co-stimulation.

3). Co-stimulation with either B7-1 or CD58 is both able to further enhance ERK-2 activity and strongly activate the p38 MAPK pathway, but only B7-1 co-stimulation induces high levels of JNK-1 activity (227).

Human B7-H3 has been recently identified as a new co-stimulatory member of the B7 family. It is expressed on GM-CSF-stimulated monocytes and IFN-γ–activated dendritic cells, as well as on $CD3^+$ T cells activated with PMA and ionomycin (228). B7-H3–Ig fusion protein can co-stimulate proliferation of $CD4^+$ and $CD8^+$ T cells through another receptor than CD28 and ICOS; the nature of this receptor is currently undetermined.

1.3.1.3 The TNF family

Several members of the tumor–necrosis factor receptor superfamily can also deliver positive co-stimulatory signals both during early and late stages after TCR ligation with antigen. Five co-stimulation pairs of the TNFR-TNF family members have been well recognized as positive regulators of T cell responses, i.e., OX40-OX40L, 4-1BB-4-1BBL, CD30-CD30L, CD27-CD70 and HVEM-LIGHT (193). Moreover, some members of the TNF ligand family,

such as FasL, CD40L, TRAIL, TRANCE and LIGHT, can reversely transduce positive co-stimulatory signals into T cells (229, 230, 231, 232, 233, 234).

Expression of OX40 (CD134) is restricted to activated T cells in humans and rodents (235, 236), while CD134L are detected on several kinds of human and murine cells, such as activated B cells (237), dendritic cells (238), and vascular endothelial cells (239). It was proposed that OX40 signals act in a temporal manner after CD28 stimulation, and allow effector T cells to survive and continue proliferating in later responses (240, 241).

4-1BB (CD137, ILA) is primarily expressed on activated $CD4^+$ and $CD8^+$ T cells (241, 242), as well as on activated NK cells (243), B cells, macrophages, dendritic cells and eosinophils (244). 4–1BBL is expressed on mature DC, and on activated B cells and macrophages; its expression can be regulated by LPS, Ig or CD40 (244, 245, 246, 247). Stimulation of 4–1BB induces higher levels of $CD8^+$ Tcell proliferation than for $CD4^+$, and appears to be critical for $CD8^+$ cell survival (248, 249).

CD30 is expressed on activated B or T cells. Expression of CD30 on T cells is dependent upon the presence of CD28 co-stimulatory signals or exogenous IL-4 during primary T cell activation (250, 251). CD30L is expressed on T and B cells, macrophages, and a variety of hematopoietic cells and tumors (252, 253). On T cells, CD30 is primarily expressed on

activated $CD8^+$ T cells, while CD30L is on activated $CD4^+$ T cells (254). Both CD30 and CD30L can affect cell activation and cell death (251, 255).

CD27 is constitutively expressed on T cells and this expression is significantly upregulated by reagents that cross-link TCR or CD3 (256, 257). It functions in T-cell activation, T-cell development, and T-cell–dependent antibody production by B cells (258, 259). CD27 is irreversibly lost from a subset of long-term repeatedly stimulated T cells, which are likely memory cells (256). The expression of CD27L (CD70) is found on medullary thymic epithelium and can be rapidly induced on both T and B cells after activation. The specific interaction of CD27 with its ligand CD70 supports clonal expansion of both antigen-stimulated CD4 and CD8 T cells, and enhances the generation of cytolytic T cells (260).

As a member of the TNF ligand family, LIGHT is constitutively expressed on "immature" dendritic cells and expressed on resting as well as activated T cells (234). It binds to three distinct members of the TNF receptor family, i.e., HVEM, LTβR and DcR3 (261, 262). LIGHT provides costimulation by triggering HVEM on T cells; such co-stimulation produces cytokines, including IFN-γ and GM-CSF, that are pivotal for Th1 type (T-helper cell type 1) immune responses, while IL-4 production is slightly increased (261, 263, 264). On the other hand, LIGHT itself can receive co-stimulatory signals and deliver "reverse signaling" to the LIGHT-bearing T cells (234, 265).

I.3.2 Inhibitory molecules

Inhibitory molecules play important roles in the regulation of TCR stimulation. It is the harmony of co-stimulatory and inhibitory signals that modulate the eventual magnitude and quality of immune responses.

The best-studied inhibitory receptor is CTLA-4 (cytotoxic T lymphocyte antigen-4, also called CD152), a member of the CD28 family. CTLA-4 binds to B7.1 and B7.2 with higher affinity then CD28. Resting T cells express little surface CTLA-4, whose expression increases upon T-cell activation. CTLA-4 inhibits T-cell proliferation and cytokine production induced by stimulation with anti-CD3 and anti-CD28 or other co-stimulatory molecules (197, 266, 267). Recent data suggest that CTLA-4 regulates T-cell signaling by attenuating TCR accumulation and retention within the immunological synapse (268).

Another inhibitory member of the CD28 family is PD-1. As with the other CD28 family members, its ligands, PD-L1 and PD-L2 are related to the B7 proteins (210, 269). PD-1 is expressed on activated T cells (270); engagement of PD-1 leads to inhibition of T-cell proliferation and cytokine production in response to anti-CD3 and anti-CD28 antibody stimulation (210, 269).

In summary, there is enormous complexity in the cell-cell interactions regulating T-cell

activation. Co-stimulatory receptors can bind to more than one ligand, and vice versa (190, 222); they may work in an synergistic (223) or antagonistic (197) way. Extensive studies lead to continued discoveries of new members of co-stimulatory and inhibitory molecules. The current known signaling molecules provide us with clues for further understanding of the molecular mechanism of T-cell responses.

I.4. The role of lipid raft in signosome and T-cell signaling

TCR and B cell receptors (BCR) bind to Ag in a highly discriminative and sensitive fashion. These receptors are complexes composed of extracellular ligand binding domains and intracytoplasmic tails that are responsible for signal transduction. On the cytoplasmic tails of these molecules, there are conserved signaling components termed immunoregulatory tyrosine activation motifs (ITAMs). These components do not have intrinsic kinase activity, but the tyrosines within the ITAMs become phosphorylated upon TCR or BCR crosslinking, due to association of TCR/BCR with cytoplasmic RTKs. It is becoming clear that TCR/BCR and their downstream signaling molecules are spacially organized on the cell membrane, and lipid rafts is essential in such organization (271, 272, 273, 274, 275, 276, 277, 278, 279).

Lipid rafts are conserved structures that exist in a variety of cell types. They are relatively ordered membrane domains, and constitute of lipid-based platforms floating in the cell membrane. Their components sphingolipid and cholesterol are usually used as markers for their detection.

In resting mature T cells, TCR is excluded from lipid rafts that contain several key

components of the TCR signaling pathway, including the Src-family kinase, Lck, and LAT (170, 280, 281, 282). Upon TCR ligation, the CD3 complex moves into rafts where the ζ chain of TCR is phosphorylated. A number of T-cell signaling molecules, including the adapter protein SLP-76 and kinase PKC, are recruited to lipid rafts following TCR activation (283, 284). The phosphorilated TCR also associates a portion of CD4 or CD8 in lipid rafts where they interact with Lck through specific protein-protein interactions (280, 281). Notably, the proteins described above may only represent a subset of the raft-associated proteins, because over 70 different raft-associated proteins have been identified in resting T cells by using recent protein identification techniques.

Lipid rafts appear necessary for T cell signaling. Structural disruption of rafts interferes with the earliest steps of T-cell activation (282). Mice deficient in acid sphingomyelinase, which is needed for the formation of lipid rafts, show defective T-cell signaling (285). In addition, raft-associated Lck is more catalytically active than Lck staying outside the rafts (286).

Whether CD28 associates with rafts is controversial. Mounting evidences indicate that CD28 may enhance TCR singling by augmenting lipid raft aggregation (287, 199), with the recruitment and activation of signaling components, such as PI3K (198, 287) and Vav (204, 206, 288), into rafts. However, a recent study on primary human T cells demonstrated that CD3/CD28 co-stimulation cannot induce lipid rafts polarization in $CD8^+$ cells (289, 290).

The composition of lipid rafts appears different in different cell populations. Compared with $CD4^+$, $CD8^+$ T cells express higher levels of a microdomain component, glycosphingolipid GM1, and have more Thy-1 (a cell surface protein) associated with lipid rafts. In addition, during the aging process, GM1 levels in $CD8^+$ T cells increase (291). Such differences may be the molecular basis of the functional difference between $CD4^+$ and $CD8^+$ cells.

The raft also shows difference between naïve and effector T cells. GM1 resides in intracellular membranes in the resting cells. The effector cells have very high levels of plasma membrane-associated GM1 (292).

Lipid rafts are also structurally unique in different stages of thymocyte development and may play an essential role in the positive selection of immature T cells in the thymus. The first rearranged chains of TCR are expressed on the surface of pre-T cells, and signals through such TCR are important for cell survival. In contrast to mature TCR that is excluded from rafts, a large number of pre-TCRs reside in lipid rafts (293, 294). The persistent presence of pre-TCR in lipid rafts during the positive selection stage might promote pre-T cell survival. At the stage of negative selection, pre-TCRs of immature T cells stay out of the rafts, and pre-TCR ligation cannot induce stable association between pre-TCR and rafts (290, 295); this further suggests that lipid raft may play a critical role in regulating thymocyte development.

I.5. The objectives of this study

Although some members of the Eph and EFN families are expressed in lymphoid organs and on some leukocytes (*Table 2* and 35, 34, 38, 6, 9, 21, 53, 52, 20, 296, 297, 298, 104, 39, 42, 80), we are at the very beginning of understanding the roles of this large family of receptor tyrosine kinases and their ligands in immune regulation. We have previously reported that EphB6 crosslinking by mAb leads to signal transduction and apoptosis of Jurkat T cells (104), as well as augmentation of normal human T-cell responses (53). The objectives of this study are to discover the roles and mechanisms of EFNB family members in T-cell development, activation and function.

II. ARTICLES

Article 1.

Ephrin B2 Induces T Cell Costimulation

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EphrinB2 Induces T Cell Costimulation[1]

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Running title: *EphrinB2 costimulates T cells*

FOOTNOTE

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ABSTRACT

Eph kinases form the largest family of receptor tyrosine kinases, and their ligands are ephrins (EFNs), which are cell surface proteins. Some Eph kinases and EFNs are expressed on T cells, B cells, and dendritic cells, but their functions in the immune system are largely unknown. In this study, we investigated the effect of EFNB2 on murine T cells. EFNB2 mRNA was expressed in the cortex of the thymus and white pulp of the spleen. At the protein level, it was expressed on T cells and monocytes/macrophages, but not on B cells. EFNB2Rs were expressed mainly on T cells. Solid-phase EFNB2 along with suboptimal anti-CD3 strongly stimulated T cell proliferation, with concomitant augmentation of IFN-γ but not IL-2 or IL-4 secretion. The activity of cytotoxic T cells was also significantly enhanced in the presence of solid-phase EFNB2. These results indicate that EFNB2R cross-linking results in costimulation of T cells. EFNB2Rs were normally scattered on the T cell surface; after TCR cross-linking, they rapidly congregated to capped TCR complexes and then to patched rafts. This provides a morphological base for EFNB2Rs to participate in T cell costimulation. We also demonstrated that EFNB2R signaling led to augmented p38 and p44/42 mitogen-activated protein kinase activation. Our study shows that EFNB2 plays important roles in immune regulation.

Key words: EphrinB2, Eph kinases, T cells, costimulation

INTRODUCTION

The Eph family of receptor tyrosine kinases (RTK) is the largest among the RTK families, comprising 25% of known RTKs (1). According to sequence homology, Eph family members are divided into EphAs (EphA1 to 9) and EphBs (EphB1 to 6) (1) (http://cbweb.med.harvard.edu/eph-nomenclature/cell_letter.html). Their ligands are cell surface molecules called ephrins (EFNs) (1). EFNs are classified into two subfamilies. There are six members in the EFNA subfamily (EFNA1 to 6), and they are GPI-anchored cell surface proteins (1, 2) (http://cbweb.med.harvard.edu/eph-nomenclature/cell_letter.html). The EFNB subfamily consists of three members (EFNB1 to 3), and they are transmembrane proteins (1, 2). Although they are ligands, EFNs, especially EFNB subfamily members, can reverse transduce signals into cells (2, 3). The interaction between Ephs and EFNs is not very strict: one Eph can bind to several different EFNs and vice versa. However, in general, EphAs only interact with EFNAs, and EphBs, with EFNBs (2, 3). Human genome sequences have revealed 14 Eph entries and 8 EFN entries (4). Therefore, most of the Ephs and their ligand EFNs probably have already been identified.

Because both Eph receptor kinases and their ligands are cell surface molecules, they can interact only with each other if expressed on adjacent cells. Not surprisingly, these receptors and ligands are known to control accurate spatial patterning and cell positioning. Many of these findings are derived from studies in the CNS, where most Eph kinases have high-level

expression (3, 5, 6, 7, 8, 9). It has also been found that EFNB2 and its receptor EphB4 are involved in angiogenesis (10), and such a function is consistent with the known roles of Eph kinases in controlling spatial structure formation.

The expression of some Ephs and EFNs in immune cells has been documented. For example, EphA1 (11), EphA2 (12), EphA3, EphA4 (13), EphB2 (14), EphB4 (15), and EphB6 (16) are expressed in the thymus; EphB6 is expressed on mature T cells (17, 18); EphA3 is expressed in pre-B cell lines (11), and EphA4 and EphA7 are significantly expressed in B cells (19); EphA2 (19) and EphB1 (20) are expressed in certain types of dendritic cells; and some Ephs, such as EphA3 (21), EphB4 (22), and EphB6 (18, 23), are expressed in leukemia cells. As for EFNs, EFNA1 (24), EFNA3, EFNB1 (25), EFNA2, EFNA4, and EFNA5 (13) are expressed in the thymus; EFNA4 can be detected in peripheral T and B cells (19).

However, we have very limited knowledge about the function of Eph and EFN in the immune system, and publications in this area are numbered. We have recently reported that EphB6, although it lacks intrinsic tyrosine kinase activity due to a mutation in its kinase domain (16), is able to transduce signals into T cells, probably via adaptor proteins such as Cbl, Grb2, and CrkL (23), and via EphB1 with which it associates (26). $EphB6^{+}$ Jurkat cells cross-linked with anti-EphB6 mAb undergo Fas-mediated apoptosis (23). Further detailed study showed that mAb against EphB6 costimulates normal human T cells, in terms of p38 mitogen-activated protein kinase (MAPK) activation, lymphokine secretion, and proliferation (17).

Initial examination of EphB6$^{-/-}$ mice shows no gross anomaly in the thymic structure and thymocyte populations (27), suggesting compensatory mechanisms at work. Munoz et al. (13) have reported that a few soluble EphAs and EFNAs interfere with T cell development in thymic organ culture.

In this study, we investigated the immune regulatory role of EFNB2, which is a ligand of several EphB kinases, including, but not restricted to, EphB6. The EphBs binding to EFNB2 are collectively termed EFNB2Rs in this study.

MATERIALS AND METHODS

In situ hybridization

A 656-bp cDNA fragment of mouse EFNB2 cDNA from positions 29 to 684 (accession no. NM010111) was fetched with PCR from a mouse embryonic tissue cDNA library and cloned into pGEM-4Z (Invitrogen, San Diego, CA). The resulting construct pGEM-4Z-mB2 was used to transcribe antisense probes with SP6 RNA polymerase or to transcribe sense probes with T7 RNA polymerase, using digoxigenin (DIG) RNA labeling kits (Roche Diagnostics, Laval, Quebec, Canada). Mouse thymus and spleen were embedded in optimal cutting temperature compound, sectioned at 7-µm thickness, and kept at -80°C until tested. The sections were thawed for 5 min at 55°C and fixed in 4% paraformaldehyde in PBS for 10 min at 4°C. After rinsing in PBS (pH 7.4) for 2 min, the sections were immersed in 0.2 N HCl for

10 min at room temperature, briefly washed three times in PBS, and digested with 1 μg/ml proteinase K in 50 mM EDTA and 100 mM Tris (pH 8) at 37°C for 30 min. Proteolysis was arrested by immersing the slides in 4% paraformaldehyde in PBS for 5 min at 4°C, followed by three 2-min washes in PBS. After the above pretreatments, the slides were incubated in a solution containing 50% deionized formamide and 2x SSC (1x SSC: 150 mM NaCl, 15 mM trisodium citrate (pH 7.0)) at 37°C for 30 min. The slides were exposed to 40–60 μl of hybridization mixture containing 1.5–3 ng/μl DIG-labeled antisense or sense RNA probes in hybridization buffer (50% deionized formamide, 2x SSC, 0.1% SDS), heated at 55°C for 5 min, and then incubated at 42°C for 16–18 h in a chamber humidified with 50% formamide in 2x SSC. Finally, the slides were washed twice in 4x SSC, once in 2x SSC, and once in 1x SSC for 15 min each. Signals were detected by alkaline phosphatase-conjugated anti-DIG Ab, with 5-bromo-4-chloro-3-indolyl phosphate as substrate and 4-nitroblue tetrazolium chloride as chromogen, according to instructions for the use of DIG detection kits (Roche Diagnostics). The sense probe was taken as a negative control. As additional negative controls, one slide for each experiment was treated with RNase A before hybridization to deplete mRNA, one slide was hybridized with omission of the probes, and one slide was detected with omission of the primary Ab during the DIG detection procedure. These additional negative controls revealed no signals, and photographs of them are not shown.

Generation of mouse EFNB2-Fc

The coding sequence of the extracellular domains of mouse EFNB2 from positions 29 to 684

was cloned in-frame upstream of the human IgG1-Fc coding sequence in an expression vector pCMVhFc. The constructs and pcDNA3 were then transfected into CHO/dhfr- cells with Lipofectamine (Invitrogen, Burlington, Ontario, Canada). The cells were cultured in selection medium (αMEM without ribonucleosides and deoxyribonucleosides containing 5% dialyzed FCS, 0.01 mM methotrexate, 0.8 mg/ml G418, and 0.1 mg/ml gentamicin). After 2 wk of culture, well-isolated clones were handpicked and expanded in the selection medium without G418. The culture supernatants were assayed by ELISA for human IgG-Fc-positive clones, which were then expanded. Fusion proteins were isolated from supernatants of the positive clone by protein A columns, then analyzed with 10% SDS-PAGE to confirm their molecular sizes, and verified by N-terminal peptide sequencing (Sheldon Biotechnology Center, McGill University, Montreal, Canada).

Lymphocyte preparation and culture

Cells were flushed out from the BALB/c mouse spleen, and RBCs were lysed with 0.84% NH4Cl, as described elsewhere (28). The resulting cells were referred to as spleen cells. Splenic T cells were purified by deleting mouse IgG (H+L)-positive cells from spleen cells with T cell columns according to the manufacturer's instructions (Cedarlane, Hornby, Ontario, Canada). In some experiments, the T cells were fractionated from spleen cells into $CD4^+$ and $CD8^+$ cells using magnetic beads (Miltenyi Biotec, Auburn, CA). The cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and penicillin-streptomycin. Solid-phase EFNB2 and anti-CD3 were prepared by coating 96-well Costar 3595 plates (Costar, Cambridge, MA) overnight with anti-mouse CD3 (clone 2C11) in PBS

at 4°C, followed by incubating EFNB2-Fc or normal human IgG (NHIgG; as a control; Southern Biotechnology, Birmingham, AL) of different concentrations at 37°C for 2 h. The plates were finally incubated on ice for 1–2 additional hours before use.

Flow cytometry

Flow cytometry was used for measurement of EFNB2R expression as well as EFNB2 expression in different cell populations. BALB/c spleen cells were stained with EFNB2-Fc/goat anti-human IgG-PE or with goat anti-EFNB2 (R&D Systems, Minneapolis, MN)/donkey anti-goat IgG-PE (Cedarlane). For the second color, anti-Thy1.2-FITC (Caltag Laboratories, Burlingame, CA), anti-B220-FITC (clone RA3-6B2), and anti-F4/80-FITC (clone CI:A3-1) were used. To measure the expression of activation markers on T cells, splenic T cells were stained with anti-Thy1.2-PE in combination with anti-CD25-FITC (clone M-A251), anti-CD44-FITC (clone IM7), anti-CD54-FITC (clone HA58), or anti-CD69-FITC (clone FN50). All of these mAbs were from BD PharMingen (San Diego, CA), unless indicated otherwise.

[^{3}H]-thymidine uptake assay

Splenic T cells were cultured in 96-well Costar plates coated with different mAb or recombinant proteins, and [^{3}H]thymidine uptake was measured, as described previously (29).

Cytotoxic T-cell assay

The assay was performed as detailed earlier (30). Briefly, C57BL/6 or transgenic 2C mouse spleen cells (H-2^b with most of their T cells specific to L^d; 0.4 x 10^6 cells/well) were stimulated with an equal amount of mitomycin C-treated BALB/c mouse spleen cells (H-2^d) in flat-bottom 96-well plates, which were precoated with goat anti-human IgG (5 μg/ml) followed by EFNB2-Fc or NHIgG coating (both at 10 μg/ml). The cells were cultured in the presence of 10 U/ml IL-2 for 6 days. On day 6, cells receiving the same treatment were pooled and counted, and their CTL activity was measured by a standard 4-h ^{51}Cr release assay, using ^{51}Cr-labeled P815 cells (H-2^d) as targets at different E:T ratios. The lysis percentage of the test sample was calculated as follows:

% lysis = (cpm of the test sample - cpm of spontaneous release)/(cpm of maximal release - cpm of spontaneous release).

Cytokine measurement

Culture supernatants of splenic T cells placed in anti-CD3- and/or EFNB2-Fc-coated wells were harvested 1–3 days after initiation of culture. IL-2, IL-4, and IFN-γ in the supernatants were quantified by ELISA (R&D Systems) according to the manufacturer's instructions.

Laser scanning confocal microscopy

Five million BALB/c splenic T cells were first blocked with 100 μl of PBS containing 2% BSA on ice for 30 min. Five micrograms of EFNB2-Fc and 1 μg of biotinylated anti-CD3

(clone 2C11; hamster mAb) were then added to the cell suspension, which was incubated for another 30 min on ice. After washing with cold PBS, the cells were reacted with goat anti-hamster IgG (5 μg/sample) for 30 min on ice. The cells were washed with cold PBS and transferred to 100 μl of warm PBS to start the cross-linking process at 37° C. The cells were then immediately fixed with 2 ml of 3.7% formalin at room temperature for 10 min. For TCR and EFNB2R staining, the cells were reacted with streptavidin-Alexa Fluor 594 (1 μg/sample) and goat anti-human IgG-Alexa Fluor 488 (1 μg/10^6 cells) on ice for 30 min. For raft and EFNB2R staining, the procedure was similar to that for TCR and EFNB2R staining, but cholera toxin-Alexa Fluor 594 (0.5 μg/sample) was used in place of streptavidin-Alexa Fluor 594. The stained cells were then washed with PBS and mounted on slides with Prolong antifade mounting medium (Molecular Probes, Eugene, OR). The slides were examined under a confocal microscope. Digital images were processed with Photoshop (Adobe, Seattle, WA).

Immunoblotting

Twelve-well plates were coated overnight with anti-CD3 (0.8 μg/ml, 500 μl/well) at 4°C. After washing, the wells were incubated with EFNB2-Fc or NHIgG (both at 10 μg/ml, 500 μl/well) at 37°C for 1–2 h, and then at 0°C for another 2 h. BALB/c splenic T cells were seeded in the precoated plates at 5 x 10^6 cells/well, and the plates were centrifuged at 228 x g for 5 min to achieve rapid contact between the cells and the bottom of the culture wells. The cells were then cultured at 37°C for 2 h before being harvested. The remainder of the

procedure has been detailed in our previous publication (28). Briefly, the harvested cells were washed and lysed in lysis buffer for 10 min; the cleared lysates were resolved in 10% SDS-PAGE with 50 μg of protein/lane and were then blotted onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with rabbit anti-phospho-p38 MAPK Ab followed by rabbit anti-p38 MAPK Ab, or with rabbit anti-phospho-p44/42 MAPK Ab followed by anti-p44/42 MAPK. All of the Abs used in immunoblotting was from New England Biolabs (Mississauga, Ontario, Canada). Signals were revealed by ECL.

RESULTS

Expression of EFNB2 in the thymus and spleen according to in situ hybridization

The BALB/c mouse thymus and spleen were examined by in situ hybridization using DIG-labeled EFNB2 antisense and sense probes. As shown in Fig. 1, EFNB2 was prominently expressed in the thymic cortex and spleen white pulp. This expression pattern suggests that EFNB2 might have a function in lymphocytes cells, particularly T cells.

EFNB2 and EFNB2R expression in T cells, B cells and monocytes/macrophages according to flow cytometry

We first examined EFNB2 expression (Fig. 2A). Splenic T cells were double-labeled with anti-CD3/anti-EFNB2 and analyzed by flow cytometry. Cells with EFNB2 intensity above

the control goat IgG were referred to as EFNB2-positive cells. In freshly prepared cells, 7.1% T cells were EFNB2 positive. The T cells rapidly up-regulated their EFNB2, and EFNB2-positive T cells reached 23.2% after overnight culture in medium. The expression of EFNB2 on CD4 and CD8 cells was similar (Fig. 2C); after overnight culture, 26.1 and 28.8% of CD4 and CD8 cells, respectively, became EFNB2 positive. Activation of $CD3^+$ T cells by anti-CD3 or anti-CD3 plus anti-CD28 (both on solid phase) for 24–48 h did not augment EFNB2 expression, as shown in Fig. 2D; actually, there was a moderated decrease of $EFNB2^+$ cells at 24 h after the activation. EFNB2 was minimally expressed in freshly isolated or cultured B cells ($B220^+$) (0.2 and 4.9%, respectively) (Fig. 2A). Monocytes/macrophages ($F4/80^+$) were another cell population showing significant expression of EFNB2 after culture, with 5.2% positive in freshly isolated ones and 31.2% positive after culture (Fig. 2A).

Next, we examined EFNB2R expression on these cells (Fig. 2B). EFNB2Rs were predominantly expressed on T cells, with 8.6% positivity in freshly isolated T cells and 42.9% positivity in cultured ones. Both CD4 and CD8 cells expressed EFNB2R, but more CD4 cells expressed EFNB2R than CD8 cells after overnight culture (47.9 vs 28.9%) (Fig. 2C). The expression of EFNB2R was not significantly affected by the status of T cell activation; T cells activated by solid-phase anti-CD3 or anti-CD3 plus anti-CD28 for 24 or 48 h had no increase in EFNB2R expression, but a moderate decrease of the expression could be consistently observed at 24 h (Fig. 2D). B cells had minimal receptor expression either before (data not shown) or after culture (Fig. 2B). Some monocytes/macrophages (16.9%) become

EFNB2R positive after culture (Fig. 2B).

The low expression of EFNB2 and EFNB2R in freshly isolated T cells and their subsequent up-regulation after culture was intriguing. We suspected that some serum factors were responsible for this phenomenon. To test this possibility, we cultured the T cells overnight in 100% fresh untreated mouse serum as well as in 100% heat-inactivated mouse serum. As shown in Fig. 2E, T cells ($CD3^+$) cultured in the former but not the latter failed to up-regulate both EFNB2 and EFNB2R. It is probable that heat-sensitive serum proteases rapidly cleave EFNB2 and EFNB2R from the cell surface. Such cleavage is not unusual for many cell surface molecules, including several TNF family members. Alternatively, a heat-sensitive factor(s) in serum might be suppressive of EFNB2 and EFNB2R expression.

These results revealed the expression pattern of both EFNB2 and EFNB2R in T cells, B cells, and monocytes/macrophages, and suggest that EFNB2 might have an important effect on T cells in the form of either T cell-T cell collaboration or T cell-monocyte/macrophage interaction during Ag presentation.

EFNB2 enhances T-cell proliferation and modulates activation marker expression

The predominant expression of EFNB2R on T cells led us to investigate whether EFNB2 could modulate T cell function. For this purpose, both anti-CD3 mAb and EFNB2-Fc were put on solid phase. Anti-CD3 (at a suboptimal concentration) or EFNB2-Fc (at an optimal

concentration) alone caused negligible T cell proliferation (Figs. 3, B and C), but EFNB2-Fc dose-dependently induced their proliferation in the presence of anti-CD3 (A). Next, T cells were cultured in wells coated with an optimal amount of EFNB2-Fc and various amounts of anti-CD3. As shown in Fig. 3B, EFNB2-Fc but not NHIgG (a negative control for EFNB2-Fc) augmented T cell proliferation when anti-CD3 was used at different concentrations. This result suggests that EFNB2R cross-linking reduces the T cell response threshold, and that EFNB2-expressing cells might be able to costimulate T cells. We also compared the costimulation by EFNB2-Fc with that by anti-CD28 mAb (Fig. 3C). The magnitude of costimulation mediated by EFNB2R was lower than the classical costimulating molecule CD28. $CD4^+$ cells and $CD8^+$ cells were both sensitive to EFNB2 costimulation, because their proliferation was similarly augmented by solid-phase EFNB2-Fc in the presence of suboptimal anti-CD3 (Fig. 3D).

The T cell response to EFNB2 was also assessed by their expression of activation markers. T cells were stimulated with solid-phase anti-CD3 alone (at a suboptimal concentration) or solid-phase anti-CD3 plus EFNB2-Fc (at an optimal concentration). As shown in Fig. 4, anti-CD3 alone (shaded area) at such a concentration was not able to up-regulate the activation markers examined, i.e., CD25, CD44, CD54, and CD69. After combined stimulation with anti-CD3 and EFNB2-Fc, CD69 but not CD25, CD44, or CD54 was significantly up-regulated. In contrast, the classical costimulation by anti-CD28 drastically enhanced all of the activation markers examined, and CD69-positive T cells reached 61.5%,

which was 2-fold higher than that of EFNB2-Fc-costimulated cells. The results indicate that EFNB2R can costimulate T cells, but this is quantitatively and qualitatively different from CD28.

EFNB2 enhances T-cell effector functions

We next examined the effect of EFNB2 on T cell effector functions, such as lymphokine production and CTL activity.

Again, T cells were stimulated with solid-phase anti-CD3 alone (at a suboptimal concentration) or in combination with EFNB2-Fc or anti-CD28 (both at optimal concentrations and on solid phase). NHIgG was used as a negative control for EFNB2-Fc. Anti-CD3 alone did not trigger lymphokine production. Anti-CD28 costimulation drastically induced IL-2, IL-4, and IFN-γ, as expected (Fig. 5). However, EFNB2-Fc costimulation augmented only IFN-γ but not IL-2 or IL-4 release. The IFN-γ level stimulated by EFNB2-Fc was lower than, but of the same order of magnitude of that induced by anti-CD28 costimulation. The lack of IL-2 and IL-4 production was not due to a shift of secretion kinetics during EFNB2 costimulation, because no production of these lymphokines was observed during any time between days 1 and 3 after culture. This again demonstrated the qualitative difference between costimulation mediated by CD28 and EFNB2R.

In EFNB2-Fc-coated wells, H-2^d (BALB/c)-stimulated CTL development of the C57BL/6 T cells (Fig. 6A) or L^d-specific 2C T cells (B) was greatly enhanced, whereas control NHIgG

had no such effect. As 2C T cells are predominantly CD8 cells (>85% Thy1.2$^+$ cells were CD8 cells, and <3% Thy1.2$^+$ cells were CD4$^+$ cells), our result suggests that EFNB2 can directly costimulate CD8 cells to achieve CTL differentiation with little dependence on CD4 help. This result is consistent with the fact that CD8 cells in the absence of CD4 cells could be directly costimulated to proliferate by solid-phase EFNB2-Fc (Fig. 3D). The costimulatory effect could be observed only when EFNB2-Fc was on the solid phase in the beginning of the culture. Soluble EFNB2-Fc added to the culture on the last day before the CTL assay or during the CTL assay had no effect (data not shown). This is in agreement with the notion that EFNB2 provides costimulation, which is an early event during T cell activation and differentiation.

The results of this section demonstrate that EFNB2 can selectively enhance certain T cell effector functions, likely by promoting costimulation to T cells during their early stage of activation.

EFNB2 triggers signaling events in T cells

To understand the molecular basis of EFNB2 costimulation, we examined translocation of EFNB2Rs and TCR, and their relationship with lipid rafts on T cell membranes immediately after TCR cross-linking. T cells were preincubated with anti-CD3-biotin, followed by a second Ab on ice. CD3 cross-linking started when the cells were transferred to 37°C. The TCR complex was stained by streptavidin-Alexa Fluor 594 in red; EFNB2Rs were stained by

EFNB2-Fc followed by anti-human IgG-Alexa Fluor 488 in green; and the lipid rafts in the T cell membrane were stained by cholera toxin-Alexa Fluor 594 in red. In resting T cells, rafts, TCR, and EFNB2Rs were distributed throughout the cell surface as small speckles. After 10-min cross-linking with anti-CD3, TCR rapidly polarized and formed a cap in one end of the cell. EFNB2Rs also congregated, and they colocalized with TCR (Fig. 7A). Such cocapping lasted >20 min (data not shown). After CD3 cross-linking, rafts underwent congregation and formed caps, but this process was slower than TCR capping and was completed at 20 min (Fig. 7B). EFNB2R congregation preceded raft congregation, but eventually at 20 min, EFNB2Rs translocated into the raft caps, suggesting that EFNB2Rs, probably like the TCR complex, only transiently associated with the rafts during TCR activation. Taken together, these data indicate that TCR and EFNB2Rs first cocap, and then both congregate to a raft cap on the cell surface after TCR-cross-linking. This provides a morphological base for EFNB2 to enhance TCR signaling, because now both TCR and EFNB2Rs are closely associated and located in aggregated rafts, which are scaffolds accommodating many signaling molecules.

Next, in EFNB2-costimulated T cells, we examined MAPK activity, which is modulated in other cell types when some Eph kinases are activated (31, 32, 33, 34). As shown in Fig. 8A, a combination of solid-phase EFNB2-Fc and suboptimal anti-CD3 stimulation for 2 h led to increased p38 MAPK and p44/42 MAPK phosphorylation, a sign of their activation, whereas anti-CD3 at suboptimal concentration had little effect, according to immunoblotting. The

membranes were reprobed with anti-p38 MAPK Ab or anti-p44/42 MAKP Ab, respectively, and the total protein levels of these kinases in all of the lanes of their respective membranes were similar. Therefore, these MAPK were activated after EFNB2R-cross-linking, and such activation might be part of the EFNB2 costimulation program. To assess the relevance of the EFNB2-up-regulated MAPK activity in T cell activation, p38 MAPK- and p44/42 MAPK-specific inhibitors, SB203580 and PD98059, respectively, were used. As shown in Fig. 8B, both inhibitors, but not their nonfunctional structural analog SB272474, inhibited EFNB2-enhanced T cell proliferation. This indicates that up-regulation of the MAPK activity is an essential part of EFNB2 costimulation signaling.

DISCUSSION

In this study, the novel functions of EFNB2 on T cell activities were revealed. In the immune system, EFNB2 was expressed on T cells and monocytes/macrophages, and its receptors were mainly expressed on T cells. Solid-phase EFNB2 could costimulate T cells in the presence of suboptimal TCR ligation. Such costimulation led to enhanced T cell proliferation and IFN- production. These functional studies were consistent with our previous report (17) in the human system using mAb against EphB6, which is one of the receptors EFNB2 binds. However, the current findings went significantly beyond the said report in several ways: 1) a physiological relevant molecule, i.e., mouse EFNB2, was used in this mouse system, and

thus, the results reflected not only the effect of EFNB2 through EphB6, but its overall effect through all the EphBRs it triggered, as would occur in vivo; 2) EFNB2 up-regulated CTL activity, which was a new finding and a logical one; 3) ligation of TCR resulted in rapid congregation of EFNB2Rs to rafts, to which TCR complex also translocated; and 4) EFNB2 costimulation augmented activation of not only p38 MAPK, as with anti-EphB6-stimulated human T cells, but also p44/42 MAPK.

As a general rule, an EFNB subfamily ligand associates with more than one specific EphB receptor. For EFNB2, it can bind to EphB1, -2, -3, -4 (35), and -6 (36). Among these, EphB2 (14), EphB4 (15) and EphB6 (16) are relevant to our study, because they are expressed in the T cell compartment. According to in situ hybridization (data not shown), both EphB4 and EphB6 were expressed in the same region of the lymphoid organs, i.e., thymic cortex and spleen white pulp, like EFNB2. Such colocalization creates a possibility for interactions between $EphB4^+$ and $EFNB2^+$ cells, and between $EphB6^+$ and $EFNB2^+$ cells. We are currently examining whether other EphBs are also expressed in these regions. It is quite possible that other EphBs, in addition to EphB6, are involved in mediating EFNB2-triggered T cell costimulation: soluble EphB4 moderately inhibited solid-phase EFNB2 costimulation on wild-type T cells, but such inhibition was more significant in EphB6 gene knockout T cells (data not shown), although the inhibition was still not complete. This suggests the involvement of both EphB4 and EphB6 in EFNB2-triggered costimulation, and the remainder of the uninhibited costimulation could be mediated by other EphBs.

We noticed that, in this study, EFNB2 costimulation failed to enhance CD25 and CD54 expression on T cells, whereas in our previous study, anti-EphB6 mAb was able to do so (17). If EphB6 is one of the receptors EFNB2 acted on, EFNB2 stimulation, in theory, should achieve effects no less than that achieved by anti-EphB6 mAb. How do we explain the discrepancy? First, our previous study was conducted in the human system, whereas the current one was performed in the murine system. The species difference is always a possible explanation. More likely, this might be caused by a different affinity between the mAb and the natural ligand EFNB2.

A recent crystal structure study has suggested that, before binding to its receptors, EFNB2 might form homodimers on the cell surface (37). Upon binding with EphB2 on another cell, two EFNB2 molecules and two EphB2 molecules on two different cells might form very stable tetramer complexes. It is conceivable that EFNB2 might similarly interact with other EphBs. This might be the molecular basis for EFNB2 to provide strong and lasting costimulation to T cells. Lymphoid organs are densely packed with $EFNB2^+$ T cells as well as other $EFNB2^+$ cells. EFNB2 on these cells might constantly stimulate EFNB2R on the neighboring T cells, and reduce their response threshold to foreign Ags. This is reminiscent of recent findings that, in vivo, T cells constantly receive low level stimulation from self-Ag on neighboring cells, and such stimulation promotes T cell sensitivity to the foreign Ags (38, 39). To maintain active signaling pathway downstream of EFNB2R due to the possible

constant interaction with its ligands, continuous expression of new EFNB2Rs with simultaneous shedding of the old ones might be a valid strategy. We found that T cells cultured in untreated serum had low EFNB2R and EFNB2 expression, but the expression drastically augmented when the serum was heat inactivated. This suggests that rapid shedding of these surface molecules is indeed a possibility, although this needs confirmation. With that said, we cannot exclude that this is due to serum inhibitory factors. Further investigation in this regard is warranted.

EFNB2 costimulation seems qualitatively different from anti-CD28 costimulation in that only IFN-γ but not IL-2 or IL-4 was produced in the former. In fact, similar findings were observed using anti-EphB6 mAb (17), or EFNB2 (the current study), or EFNB1 and EFNB3 (data not shown). Thus, this seems to be an intrinsic property of costimulation mediated by EphBs. Consistent with this finding, we found that solid-phase EFNB2 augmented CTL activity, for which increased IFN- γ is likely a contributing factor. Naturally, the enhanced CTL might benefit from other cellular activities that EFNB2 promoted, such as T cell proliferation, which could amplify the number of the CTL precursors.

The critical role of lipid rafts in TCR signaling has been increasingly appreciated. Some TCR signaling molecules, such as Src kinase, linker for activation of T cells, and Ras are constitutively situated in the rafts (40, 41, 42), whereas others, such as CD3ζ-associated protein-70, Vav, phospholipase C-γ1, protein kinase C-θ, and IκB kinase components

translocate into rafts after TCR triggering (43, 44, 45, 46). Such translocation enables TCR to use these molecules in the scaffold of rafts to accomplish its signaling. We studied the relationship between EFNB2R and rafts under various conditions. Cross-linking EFNB2Rs using EFNB2 in the absence or presence of suboptimal TCR cross-linking did not alter the EFNB2Rs or raft distribution, and they remained evenly scattered on T cell surface (data not shown). Only rather strong TCR cross-linking (as shown in Fig. 7) resulted in caps containing EFNB2Rs, rafts, and TCR. EFNBR2 capping had different kinetics from raft capping, suggesting that EFNB2Rs are not constitutively located in rafts. This indicates that EFNB2R belongs to the second category of signaling molecules (likely CD3ζ et al., as discussed above) that move to the rafts to assist TCR signaling only after TCR have already been triggered.

For proper T cell activation, Ras and Rac signaling pathways need to be mobilized. Ras activation leads to activation of p44/42 MAPK kinases, which, in turn, results in the synthesis and activation of various transcription factors. In contrast, activation of Rac and Cdc42 small G proteins leads to p38 MAPK activation, which is essential for cytoskeleton reorganization. Such reorganization is now known to be pivotal for the T cell signaling (47). We have found that the activities of both p44/42 and p38 MAPK are enhanced in the presence of EFNB2 costimulation, and this is consistent with the roles of these MAPKs in T cell activation. Recently, it has been reported that EphB2 activation results in inhibition of p44/42 MAPK in neuronal cells (34), and that EphA activation leads to inhibition of this kinase in several cell

lines of endothelial and epithelial origin (48). Obviously, these reports deal with cell types different from those in our study. The consequence is different as well. In neuronal cells, endothelial cells, and epithelial cells, Eph activation does not induce cell proliferation, while in T cells, it does. Further comparative studies on the EFNB2R signaling in T cells and nonimmune cells will be interesting.

We have demonstrated the costimulatory effects of EFNB2 on mouse T cells in this study. The function of Ephs and their ligands in immune regulation represents a new domain of research in immunobiology and deserves more attention.

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Fig. 1

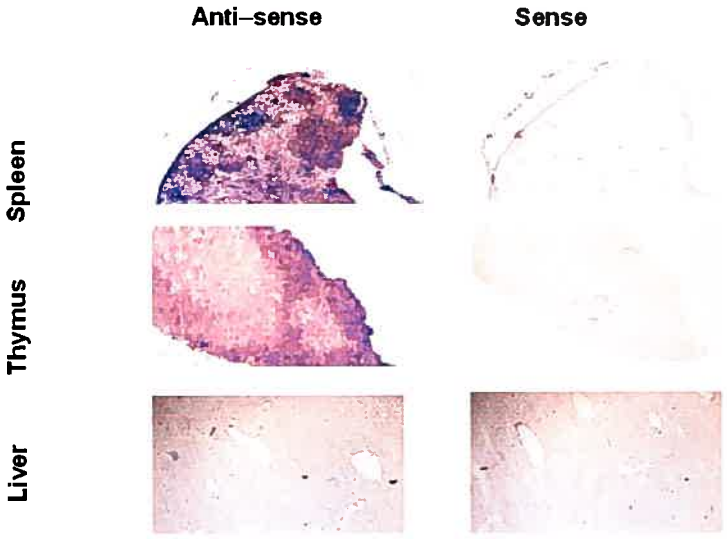
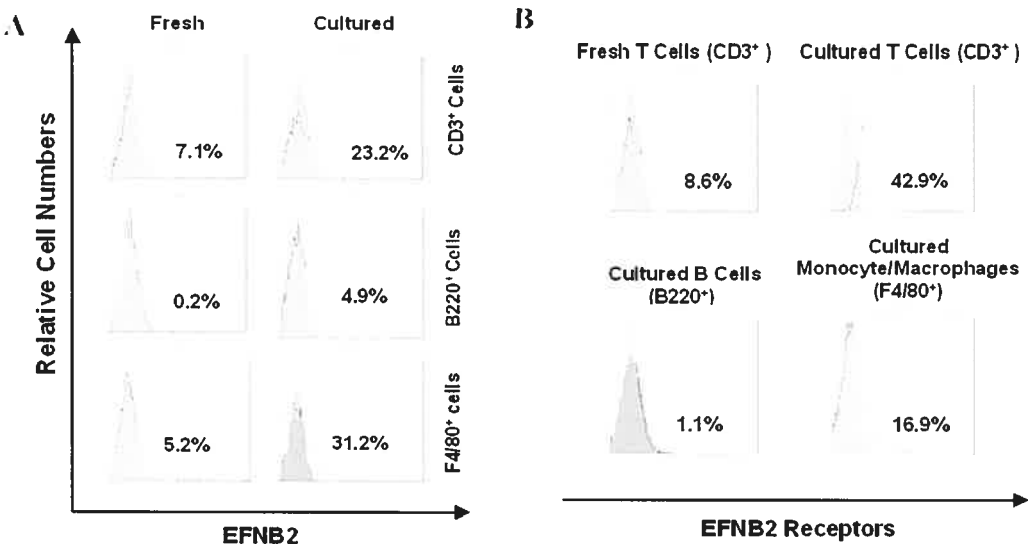
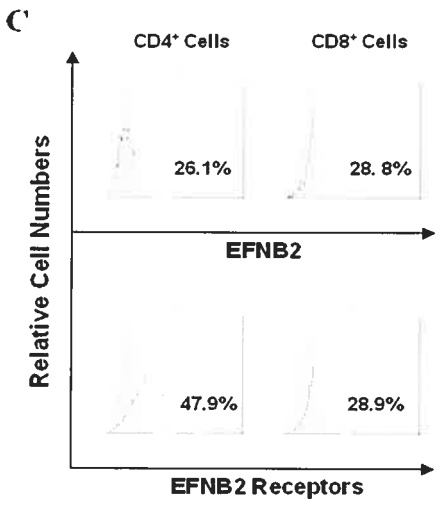


Figure 1. EFNB2 expression in the thymus and spleen according to in situ hybridization

Thymus and spleen sections were hybridized with DIG-labeled anti-sense or sense EFNB2 probes. The liver was used as a negative control. The original magnification was 100 X.

Fig. 2


A
Fresh
Cultured
Relative Cell Numbers
CD3⁺ Cells
B220⁺ Cells
F4/80⁺ cells
7.1%
23.2%
0.2%
4.9%
5.2%
31.2%
EFNB2
B
Fresh T Cells (CD3⁺)
Cultured T Cells (CD3⁺)
8.6%
42.9%
Cultured B Cells (B220⁺)
Cultured Monocyte/Macrophages (F4/80⁺)
1.1%
16.9%
EFNB2 Receptors


C
CD4⁺ Cells
CD8⁺ Cells
Relative Cell Numbers
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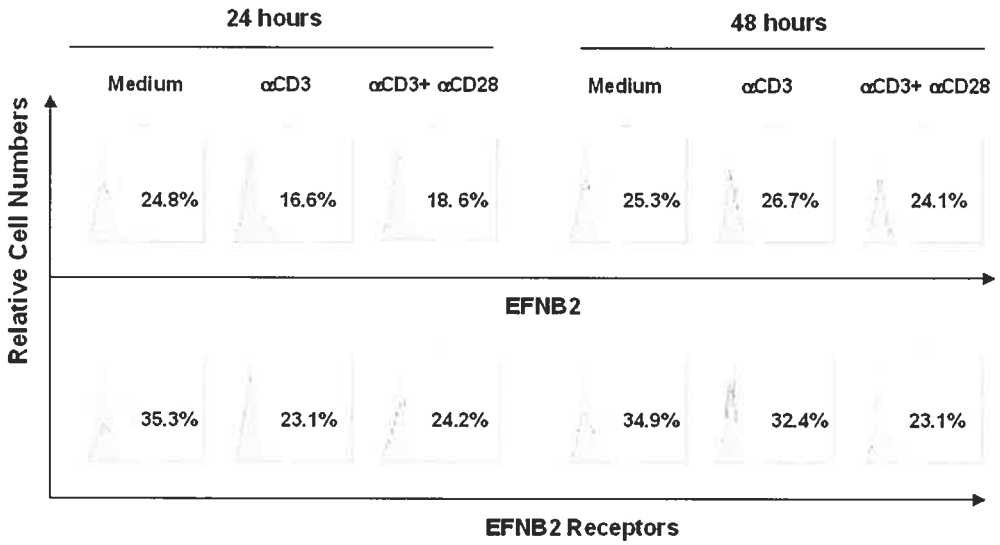
28. 8%
EFNB2
47.9%
28.9%
EFNB2 Receptors

D


24 hours
48 hours
Medium
αCD3
αCD3+ αCD28
Medium
αCD3
αCD3+ αCD28
Relative Cell Numbers
24.8%
16.6%
18. 6%
25.3%
26.7%
24.1%
EFNB2
35.3%
23.1%
24.2%
34.9%
32.4%
23.1%
EFNB2 Receptors

E

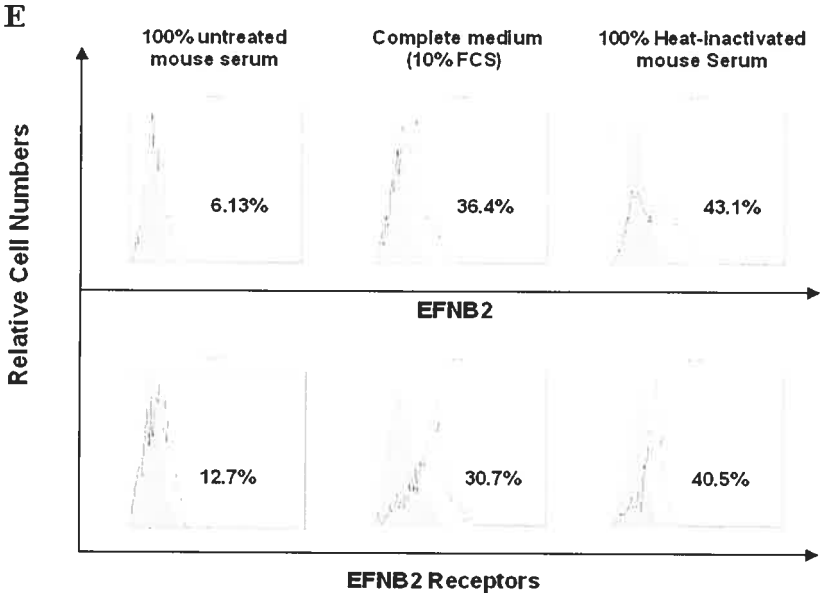

100% untreated mouse serum
Complete medium (10% FCS)
100% Heat-inactivated mouse Serum
Relative Cell Numbers
6.13%
36.4%
43.1%
EFNB2
12.7%
30.7%
40.5%
EFNB2 Receptors

Figure 2. Flow cytometry analysis of EFNB2 and EFNB2R expression on immune cells

All experiments were performed more than twice, and were reproducible. Representative results are shown. Two-color flow cytometry was used to assess EFNB2 or EFNB2R expression on T cells, B cells and monocytes/macrophages; T cells were stained with anti-CD3; B cells, with anti-B220; and monocytes/macrophages, with F4/80. One-color flow cytometry was used to assess EFNB2 or EFNB2R expression on magnetic beads-purified CD4 or CD8 cells. A: EFNB2 expression on $CD3^+$ T cells, $B220^+$ B cells or $F4/80^+$ monocytes/macrophages. B: EFNB2R expression on $CD3^+$ T cells, $B220^+$ B cells or $F4/80^+$ monocytes/macrophages. C: EFNB2 and EFNB2R expression on magnetic beads-purified CD4 or CD8 cells. D: Kinetics of EFBB2 and EFNB2R expression on $CD3^+$ T cells activated with solid phase anti-CD3 (suboptimal) or anti-CD3 (suboptimal) plus anti-CD28. E: EFNB2 and EFNB2R expression on $CD3^+$ T cells cultured in 100% untreated or heat-inactivated mouse serum. The percentages of positive cells are indicated.

Fig. 3

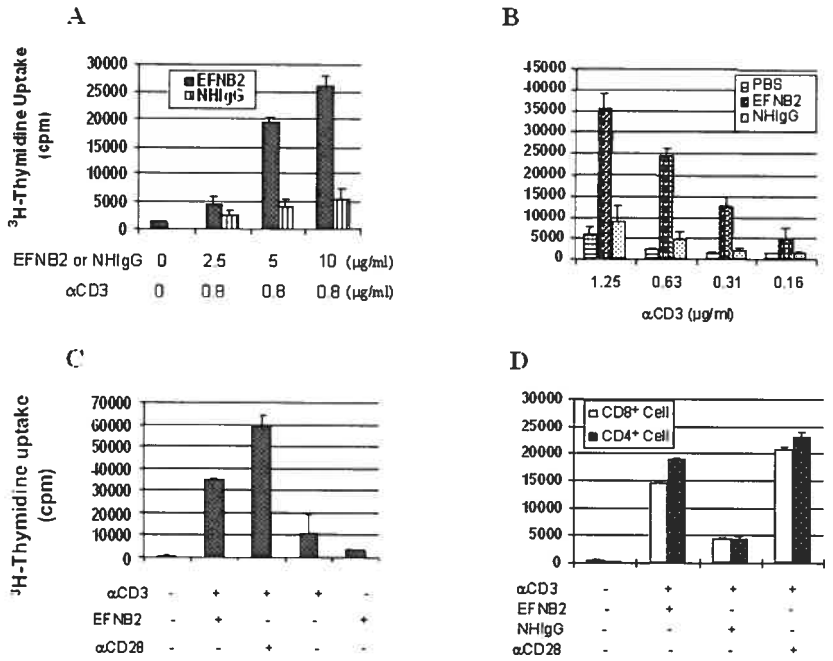


Figure 3. EFNB2R crosslinking enhances the T-cell response to TCR stimulation

All experiments were conducted more than 3 times and were reproducible. Representative results are shown. The affix "anti" used in conjunction with mAbs is simplified as "α" in this and all the other figures of this article. All the concentrations indicated in the figure legends represent those employed during the coating procedure. Normal human IgG (NHIgG) served as a control for EFNB2-Fc. In some cases, the wells were only coated with anti-CD3, followed by PBS incubation (PBS), and were used as additional blank controls. BALB/c T cells were cultured in wells coated with (A) a suboptimal amount of anti-CD3 (0.8 μg/ml) and different amounts of EFNB2-Fc; (B) a fixed optimal amount of EFNB2-Fc (10 μg/ml) and different amounts of anti-CD3; or (C) a suboptimal amount of anti-CD3 (0.8 μg/ml)

along with optimal amounts of anti-CD28 or EFNB2-Fc (both at 10 μg/ml). Magnetic beads-purified $CD4^+$ or $CD8^+$ cells were also cultured in wells coated with EFNB2-Fc, NHIgG, or anti-CD28 in the presence of suboptimal solid phase anti-CD3 (D). The cells were cultured for 48 h, and their ^{3}H-thymidine uptake in the last 16 h was measured. Means ± SD of the cpm from triplicate samples are shown.

Fig. 4

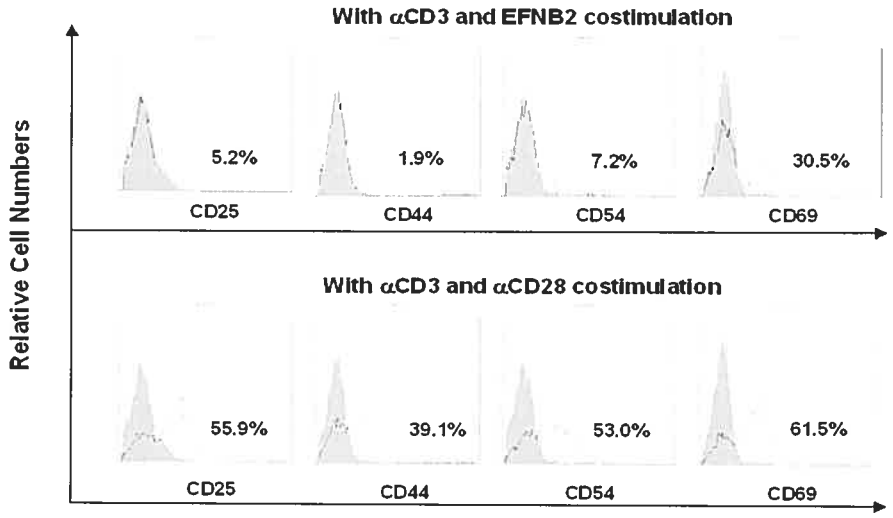


Figure 4. Activation marker expression of EFNB2-Fc-costimulated T cells

All experiments were carried out more than 3 times and were reproducible. Representative results are shown. BALB/c T cells were cultured in wells coated with anti-CD3 mAb (0.8 μg/ml) plus EFNB2-Fc or anti-CD28 (both at 10 μg/ml). Their activation marker expression at 24 h was assessed by 2-color flow cytometry, using anti-Thy1.2-PE for T-cell gating, and anti-CD25-FITC, anti-CD44-FITC, anti-CD54-FITC, and anti-CD69-FITC for activation

markers. Percentages represent activation marker positive cells among Thy1.2 positive cells (solid lines) after deducting fluorescence in cells stimulated with suboptimal anti-CD3 alone (shaded areas).

Fig. 5

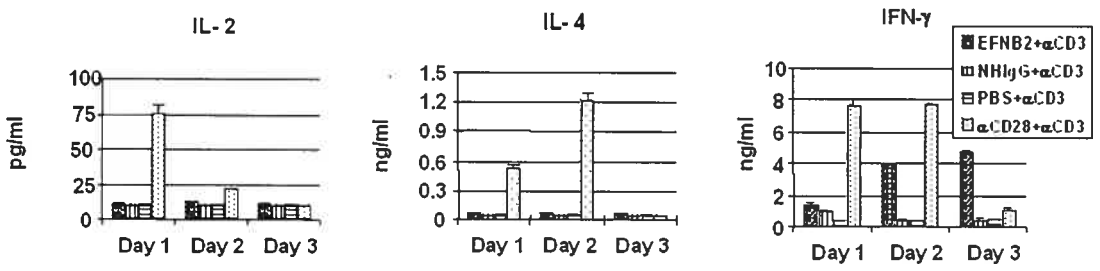


Figure 5. Solid phase EFNB2-Fc strongly augments IFN-γ but not IL-2 or IL-4 production by anti-CD3-stimulated T cells

Mouse spleen T cells were stimulated with solid phase EFNB2-Fc (10 μg/ml) in the presence of a suboptimal concentration of anti-CD3 (0.8 μg/ml). Anti-CD28 (10 mg/ml) and NHIgG were used as positive and negative controls, respectively. The culture supernatants were harvested from days 1 to 3, and cytokines in the supernatants were measured by ELISA. The experiment was performed more than twice and was reproducible. Means ± SD of

representative results are shown.

Fig. 6

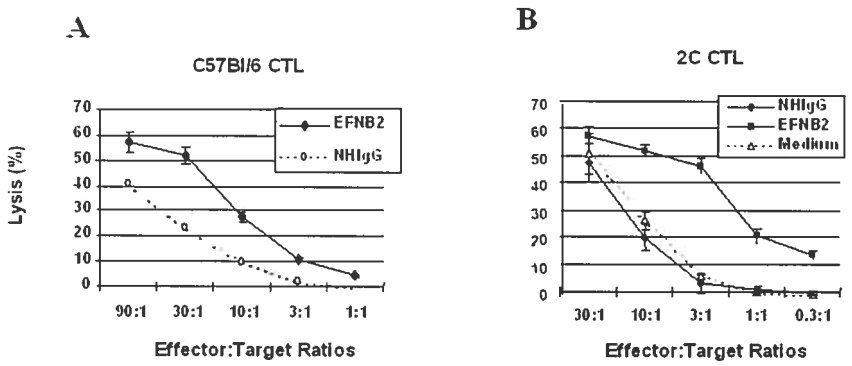


Figure 6. Effect of solid phase EFNB2-Fc on CTL development

C57BL/6 mouse (A) or 2C mouse (B) spleen cells were mixed with an equal amount of mitomycin C-treated BALB/c mouse spleen cells and seeded in flat-bottomed 24-well plates, which were pre-coated with EFNB2-FC or NHIgG (both at 10 μg/ml), or not coated (Medium). After 6 days, CTL activity in the stimulated cells was measured by a standard 4-h ^{51}Cr-release assay, using P815 cells as targets. The samples were tested in triplicate, and means ± SD of the percentage of target cell lysis are shown. The experiments were performed twice with similar results, and the data of a representative experiment are presented.

Fig. 7

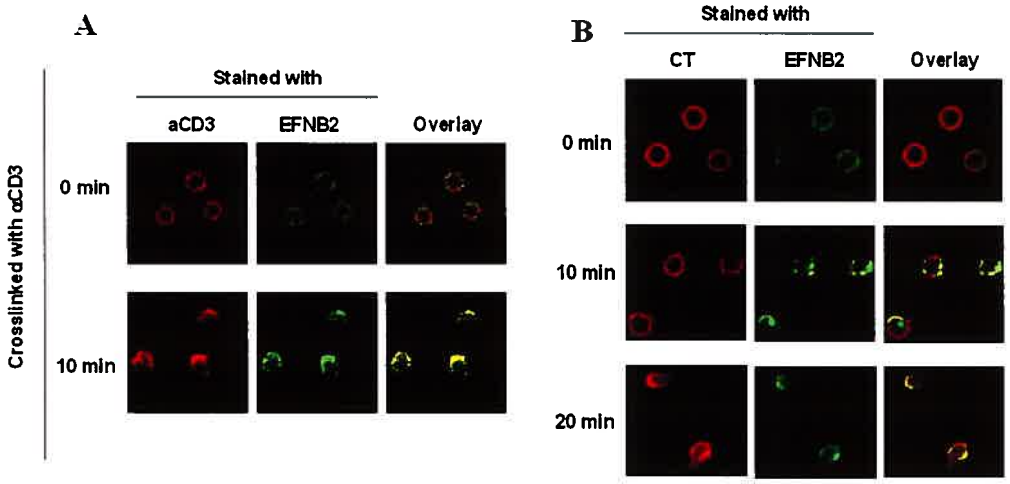


Figure 7. Rapid colocalization of EFNB2 receptors with TCR and raft caps after anti-CD3 crosslinking

BALB/c spleen T cells were crosslinked with anti-CD3 for 0, 10 or 20 min, as indicated. The locations of TCR (stained with biotin-anti-CD3 followed by Alexa Fluor 594-streptavidin in red) (A), EFNB2 receptors (stained with EFNB2-Fc followed by Alexa 488-anti-human IgG in green) (A and B), and rafts (stained with Alexa Fluor 594–cholera toxin (CT) in red) (B) were revealed by confocal microscopy. All experiments were conducted more than 3 times and were reproducible. Representative results are shown.

Fig. 8

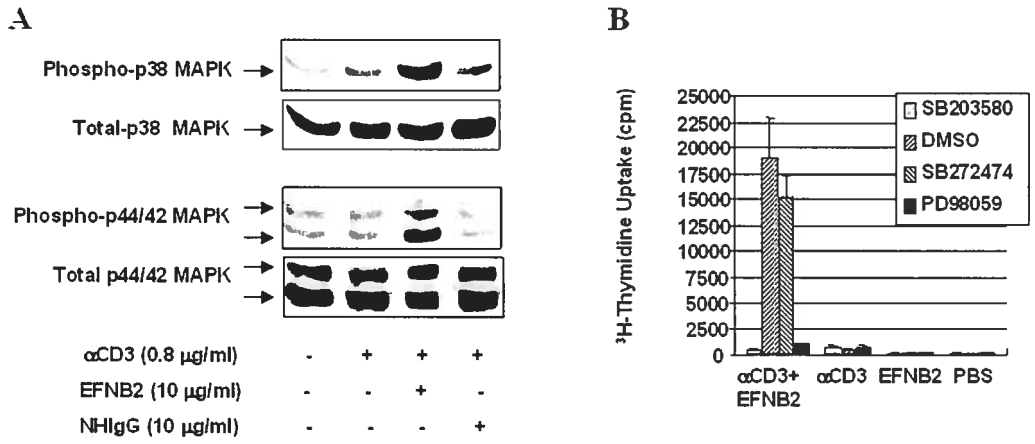


Figure 8. Activation of p38 MAPK and p44/42 MAPK in T cells by solid phase EFNB2-Fc

A. Immunoblotting of p38 and p44/42 MAPK

T cells were added to wells coated with EFNB2-Fc or NHIgG (both at 10 μg/ml) in the presence of a suboptimal amount of anti-CD3 (0.8 μg/ml). The cells were harvested after 2 h, and analyzed by immunoblotting. Arrows indicate signals of p38 phospho-MAPK and total p38 MAPK of the same membrane, and signals of p44/42 phospho-MAPK and total p44/42 MAPK of the same membrane.

B. p38- and p44/42-specific inhibitors inhibit EFNB2-costimulated T-cell proliferation

T cells were pre-incubated for 1 h in complete culture medium containing the p38

MAPK-specific inhibitor SB203580, p44/42-specific inhibitor PD98059, its non-functional structural analog SB272474 (all at 10 μM), or vehicle DMSO (dimethyl sulfoxide, 0.1%). The cells were then transferred to wells coated with EFNB2-Fc (10 μg/ml), anti-CD3 mAb (0.8 μg/ml), or both, and cultured for 48 h. ^{3}H-thymidine was added to the culture for the last 8 h, and thymidine uptake by the cells was measured.

Article 2.

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

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

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Running title: *EphrinB3 enhances T-cell responsiveness*

FOOTNOTE

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ABSTRACT

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

INTRODUCTION

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

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

recently (12, 13), in intestinal development and remodeling.

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

EXPERIMENTAL PROCEDURES

In situ Hybridization

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

the kit manufacturer.

Generation of Mouse EFNB3-Fc

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

Lymphocyte Preparation and Culture

Cells were flushed out from the BALB/c mouse spleen, and red blood cells were lysed with 0.84% NH4Cl, as described elsewhere (30). The resulting cells were referred to as spleen cells. Spleen T cells were purified by deleting mouse IgG (H+L)-positive cells from spleen cells with T-cell columns according to the manufacturer's instructions (Cedarlane, Hornby, Ontario, Canada). In some experiments, CD4 and CD8 cells were positive-selected from the spleen cells by magnetic beads (Miltenyi Biotec, Auburn, CA). The cells were cultured in

RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin. Solid-phase EFNB3 and anti-CD3 were prepared by coating 96-well Costar 3595 plates overnight with anti-mouse CD3 (clone 2C11) in phosphate-buffered saline (PBS) at 4 °C, followed by incubating EFNB3-Fc or, as a control, normal human IgG (NHIgG) (Southern Biotechnology, Birmingham, AL) of different concentrations at 37 °C for 2 h, followed by an additional 2-h incubation on ice. In some experiments, CD4 and CD8 cells were positive-selected from the spleen cells by magnetic beads (Miltenyi Biotech). For mixed lymphocyte reaction (MLR), spleen cells from BALB/c mice were treated with mitomycin C and mixed with C57BL/6 spleen cells at a 1:1 ratio (8×10^5 cells at 200 µl/well). The cells were cultured in the presence of NHIgG or Ephs (Eph A4, EphB3, and EphB4; R&D Research, Minneapolis, MN) for 5 days before being harvested.

^{3}H-thymidine Uptake Assay

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

Flow Cytometry

Flow cytometry was employed for the measurement of EFNB3 expression as well as EFNB3R expression in T-cell populations. BALB/c spleen cells were stained with goat

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

Cytotoxic T Lymphocyte (CTL) Assay

The assay was performed as detailed earlier (32) using transgenic 2C mouse spleen cells (in H-2^b background with most of their T cells specific to Ld, 0.4 x 10^6 cells/well) as responders, mitomycin C-treated BALB/c mouse spleen cells (H-2^d) as stimulators, and ^{51}Cr-labeled P815 cells (H-2^d) as targets. This system is very sensitive and has a high signal-to-noise ratio because of the high frequency of Ld-specific precursor T cells (30).

The lysis percentage of the test sample was calculated as follows:

% lysis = (cpm of the test sample - cpm of spontaneous release)/(cpm of maximal release - cpm of spontaneous release).

Cytokine Measurement

Culture supernatants of T cells placed in anti-CD3-coated and/or EFNB3-Fc-coated wells

were harvested 1–3 days after the initiation of culture. IL-2, IL-4, and IFN-γ in the supernatants were quantitated by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions.

Confocal Microscopy

Five million BALB/c T cells were reacted on ice for 30 min with 5 μg of EFNB3-Fc and 1 μg of biotinylated anti-CD3 (clone 2C11, hamster mAb). After washing, the cells were incubated with goat anti-hamster IgG (5 μg/sample) for 30 min on ice. They were then washed with cold PBS and transferred to warm PBS to start the crosslinking process at 37 °C for different time periods; this was followed by immediate fixation with 3.7% formalin. For TCR and EFNB3R staining, the cells were reacted with streptavidin-Alexa Fluor 594 (1 μg/sample) and goat anti-human IgG-Alexa Fluor 488 (1 μg/106 cells) on ice for 30 min. For raft and EFNB3R staining, the procedure was similar to that described above, but cholera toxin-Alexa Fluor 594 (0.5 μg/sample) was used in place of streptavidin-Alexa Fluor 594. The slides were examined under a confocal microscope.

Immunoblotting

Five million BALB/c T cells were reacted on ice for 30 min with 5 μg of EFNB3-Fc and 1 μg of biotinylated anti-CD3 (clone 2C11, hamster mAb). After washing, the cells were incubated with goat anti-hamster IgG (5 μg/sample) for 30 min on ice. They were then washed with cold PBS and transferred to warm PBS to start the crosslinking process at 37 °C

for different time periods; this was followed by immediate fixation with 3.7% formalin. For TCR and EFNB3R staining, the cells were reacted with streptavidin-Alexa Fluor 594 (1 μg/sample) and goat anti-human IgG-Alexa Fluor 488 (1 μg/106 cells) on ice for 30 min. For raft and EFNB3R staining, the procedure was similar to that described above, but cholera toxin-Alexa Fluor 594 (0.5 μg/sample) was used in place of streptavidin-Alexa Fluor 594. The slides were examined under a confocal microscope.

RESULTS

EFNB3 and EFNB3R Expression in Leukocytes

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

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

medium for 24 h) was determined by anti-EFNB3 or EFNB3-Fc one-color staining of magnetic bead-purified CD4 and CD8 cells (Fig. 2C). The expression of EFNB3 on CD4 and CD8 cells was similar (26.1 and 22.1%, respectively), although CD4 cells expressed more EFNB3Rs than CD8 cells (45.4 and 21.3%, respectively).

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

EFNB3 Enhances T-cell Proliferation and Modulates Activation Marker Expression

Because EFNB3Rs are expressed in a significant portion of T cells, it is likely that EFNB3 plays a role in modulating T-cell function. Solid-phase EFNB3, in the presence of solid-phase anti-CD3, was used to assess this possibility. EFNB3-Fc, but not a control protein (normal human IgG), dose-dependently induced T-cell proliferation in the presence of a suboptimal concentration of anti-CD3 (Fig. 3A). When T cells were stimulated with an optimal amount of EFNB3-Fc and various amounts of anti-CD3, they proliferated dose-dependently in response to the latter (Fig. 3B). We also compared the costimulation by EFNB3-Fc with that by anti-CD28 mAb (Fig. 3C). Costimulation mediated by EFNB3Rs was at a moderately lower level than that mediated by the classical costimulatory molecule CD28. Anti-CD3 (at a suboptimal concentration) or EFNB3-Fc (at an optimal concentration) alone caused low level or no T-cell proliferation, respectively (last two columns of Fig. 3C).

The response of CD4 and CD8 cells to EFNB3 costimulation was similar (Fig. 3D).

Interestingly, the costimulation via EFNB3R was insensitive to cyclosporin A (CyA). As shown in Fig. 3E, CyA, which inhibits $Ca2^+$ flux-dependent T-cell activation (33, 34), could effectively suppress ionomycin and PMA-induced T-cell proliferation but failed to do so for T cells activated by PMA plus solid-phase anti-CD28 or PMA plus solid-phase EFNB3. This finding suggests that CD28 and EFNB3R costimulation both utilize the $Ca2^+$-independent pathway. Next, we assessed whether the EFNB3 can augment anti-CD28 costimulation in the presence of anti-CD3. At optimal CD28 concentration (10 μg/ml for coating), solid-phase EFNB3 had no additional effect (Fig. 3F). However, at suboptimal anti-CD28 concentration (5 or 2.5 μg/ml), EFNB3 could moderately enhance the costimulation. This finding suggests again that CD28 and EFNB3R do not employ two totally separate pathways.

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

The expression of certain activation markers on T cells after EFNB3 costimulation was then

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

These results indicate that EFNB3Rs can enhance T-cell activation to TCR stimulation, but such enhancement seems qualitatively different from that of CD28.

EFNB3 Enhances Lymphokine Production and CTL Activity

We next examined the effect of EFNB3 on T-cell effector functions, such as lymphokine production and CTL activity. Again, T cells were stimulated with solid-phase anti-CD3 alone (at a suboptimal amount) or in combination with EFNB3-Fc or anti-CD28 (both at optimal amounts and on solid-phase). NHIgG was used as a negative control for EFNB3-Fc. Three representative lymphokines, IL-2, IL-4, and IFN-γ, were measured from day 1 to day 3 after stimulation (Fig. 5A). Anti-CD3 with or without NHIgG (i.e. PBS) did not trigger lymphokine production. Anti-CD28 costimulation drastically induced IL-2, IL-4, and IFN-γ, as expected. EFNB3-Fc led IFN- γ production to a level comparable with but moderately lower than that caused by anti-CD28 costimulation but did not stimulate IL-4 and IL-2

production. The lack of IL-2 and IL-4 production after EFNB3 costimulation was not caused by a shift of secretion kinetics, because no production of IL-2 or IL-4 was observed at any time between days 1 and 3 after culture. This result demonstrates that EFNB3 and anti-CD28 costimulation have qualitative differences.

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

Signaling Events in T cells Stimulated by EFNB3

To understand the mechanisms of EFNB3 costimulation, we first examined the translocation of EFNB3Rs and TCR on the T-cell surface and their relationship to membrane lipid rafts immediately after TCR-crosslinking (Fig. 6). TCR was crosslinked by biotinylated anti-CD3 followed by goat anti-hamster IgG. The TCR complex was stained by streptavidin-Alexa Fluor 594 in red; EFNB3Rs, by EFNB3-Fc followed by anti-human IgG-Alexa Fluor 488 in green; the lipid rafts in the T-cell membrane, by cholera toxin (CT)-Alexa Fluor 594 in red. In resting T cells, rafts, TCR, and EFNB3Rs were evenly distributed throughout the cell surface (0 min). After 10-min crosslinking with anti-CD3, TCR rapidly polarized and formed a cap in one end of the cell. EFNB3Rs also congregated, and they co-localized with TCR (Fig. 6A). After CD3 crosslinking, the rafts underwent congregation and formed caps as well. EFNB3Rs translocated into the raft caps (Fig. 6B). This indicated that the TCR complex (as

detected by anti-CD3), EFNB3Rs (as detected by EFNB2-Fc), and the raft (as detected by CT) all clumped together. This provides a morphological base for EFNB3Rs to enhance TCR signaling, because now both TCR and EFNB3Rs are closely associated and located in aggregated rafts, and their respective signaling pathways can interact closely.

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

DISCUSSION

We have reported that EFNB3 and EFNB3Rs were expressed in T cells and monocytes/macrophages. T cells costimulated by solid-phase EFNB3-Fc in the presence of suboptimal anti-CD3 showed enhanced proliferation and CTL activity, production of IFN-γ but not IL-2, and expression of certain T-cell activation markers, such as CD69. Upon TCR activation, EFNB3Rs translocated into the raft caps, to which TCR also congregated. Further downstream, MAPK activity was enhanced by EFNB3-Fc costimulation. These results suggest that EFNB3 and its receptors play important roles in T-cell activation and function. The inherent difficulty in studying the receptors and functions of EFNs is that each of them binds to multiple Eph family members. In the case of EFNB3, it is known to interact with EphA4 (35), EphB3 (36), and EphB/4 (37), and its interaction with additional Eph members is also possible. We attempted to identify the receptors mediating the ligation of EFNB3. Soluble EphA4, EphB3, and EphB4 were added to MLR. EphA4 and EphB4, but not EphB3, were able to partially inhibit the T-cell proliferation. This suggests that the former two might mediate the effect of EFNB3. However, as Ephs and EFNs are capable of two-way signaling, and the EphA4 and EphB4 can potentially interact with EFNs in addition to EFNB3, we cannot exclude the possibility that the observed inhibitory effect is due to the binding of EphA4 and EphB4 to certain EFNs (including EFNB3), which transduce negative signals into T cells. Therefore, the receptors responsible for the binding or functions of EFNB3 cannot be conclusively pinpointed until specific mAbs to (and conditional gene knockout

mice of) each and every Eph (especially EphBs) become available. However, our study is significant, because the effect of EFNB3 detected here reflects combinatory functions of all receptors triggered by EFNB3, and this is perhaps a true picture of what EFNB3 can achieve in vivo.

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

Because EFNB3 is expressed on T cells and monocytes/macrophages implies that all these cells can enhance responsiveness or reduce the response threshold of $EFNB3R^+$ T cells to antigens. This fact might explain the advantage for T cells to be activated in lymphoid organs where leukocytes are tightly packed. Although costimulation from antigen-presenting cells to T cells is well studied, we now can understand that the signaling between T-cells is equally important for T-cell activation. A typical proof of this concept can be found in any T-cell culture system, in which minimal cell density is normally required for optimal T-cell activation. Although autocrine is often quoted as a reason for such a requirement, interaction through cell-surface receptors and ligands on the T cells is likely a more fundamental

mechanism. Recently, Wang et al. (43) have demonstrated that LIGHT, which is a cell-surface ligand belonging to the tumor necrosis factor family member and is predominantly expressed on T cells, can stimulate T-cell activation both in vivo and in vitro through its receptor HveA on T cells. We have shown that in pure T-cell culture, the interaction between EphB6 and its T-cell surface ligand can enhance T-cell response to TCR stimulation (20). The expression of both EFNB3 and EFNB3R on T cells and the effect of EFNB3 on T-cells costimulation, as documented in this study, suggests the possibility that these two molecules are also involved in T-cell/T-cell co-operation.

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

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

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

as well.

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

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Fig. 1

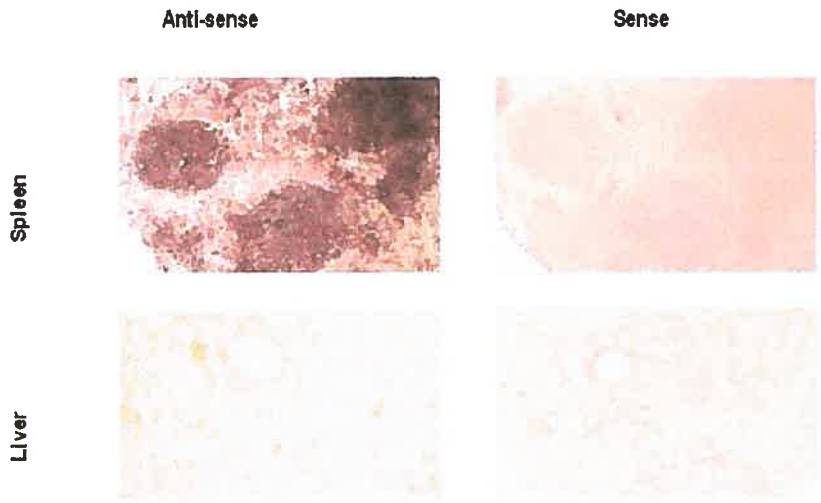

Figure 1. In situ hybridization of EFNB3

Antisense or sense probes were hybridized with BALB/c spleen sections. Liver sections served as negative controls. Original magnification: 10 X.

Fig. 2

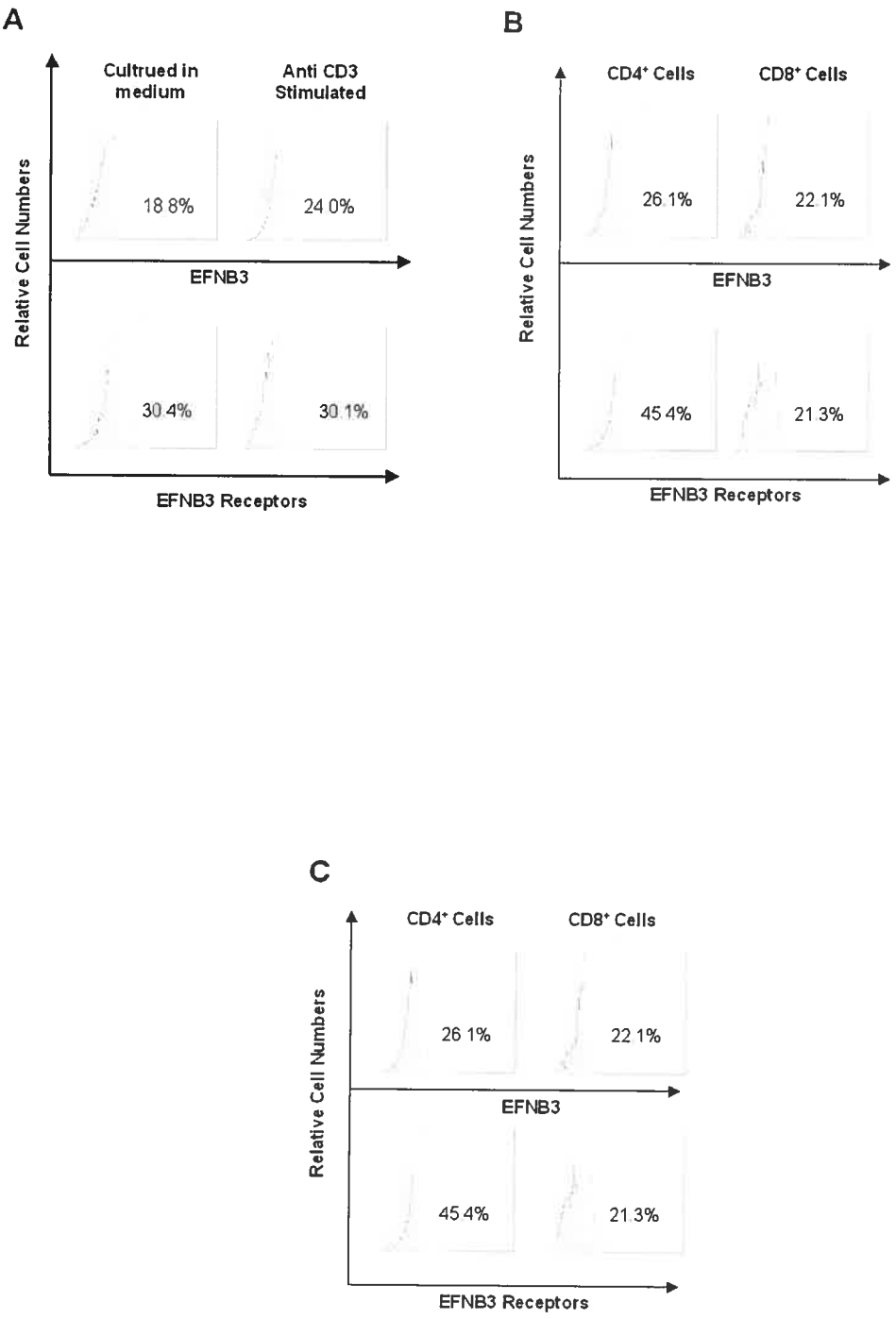
A
Cultrued in medium
Anti CD3 Stimulated
Relative Cell Numbers
18.8%
24.0%
EFNB3
30.4%
30.1%
EFNB3 Receptors
B
CD4+ Cells
CD8+ Cells
Relative Cell Numbers
26.1%
22.1%
EFNB3
45.4%
21.3%
EFNB3 Receptors
C
CD4+ Cells
CD8+ Cells
Relative Cell Numbers
26.1%
22.1%
EFNB3
45.4%
21.3%
EFNB3 Receptors

Figure 2. Flow cytometry analysis of EFNB3 and EFNB3R expression on leukocytes

A and B: Two-color flow cytometry was used to determine the expression of EFNB3 (detected with anti-EFNB3 Ab) or EFNB3R (detected with EFNB3-Fc) on T cells, B cells and monocytes/macrophages. T cells were stained with anti-CD3; B cells, with anti-B220; and monocytes/macrophages, with F4/80. For T cells analysis, the spleen cells were cultured in medium with (Activated) or without (Resting) solid-phase anti-CD3 for 24 h. For B cells and monocytes/macrophages analysis, they were assayed after 24-h culture in plain medium. C. One-color flow cytometry was used to measure the expression of EFNB3 (detected with anti-EFNB3) and EFNB3R (detected with EFNB3-Fc) on magnetic bead-purified CD4 and CD8 cells (about 95% pure) after 24-h culture in medium.

The percentages of positive cells (areas under the solid curves subtracted by shaded areas [normal rabbit IgG or Norman human IgG as controls]) are indicated. The experiment was performed more than three times and was reproducible. Results from a representative experiment are shown.

Fig. 3

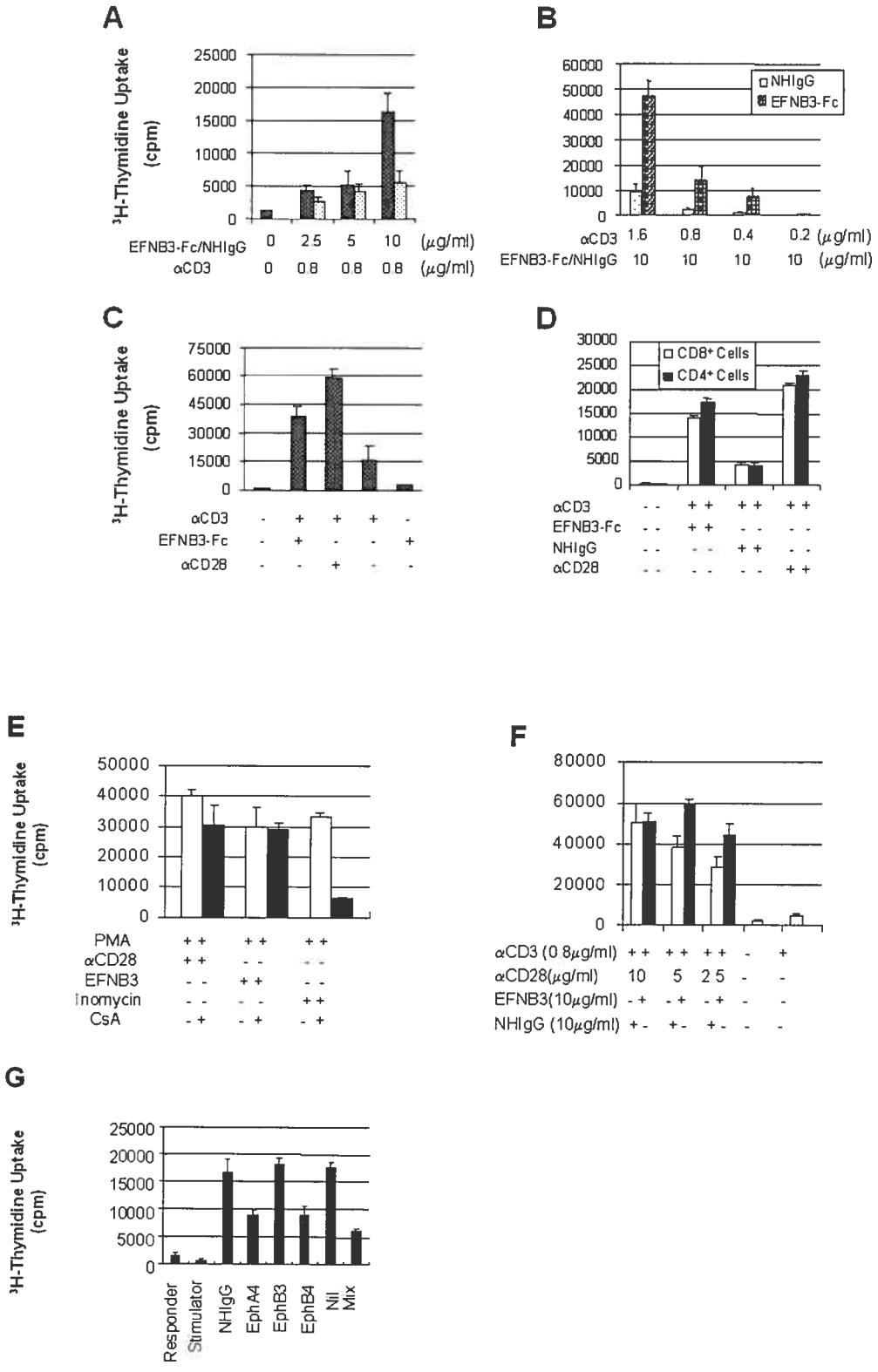
A
³H-Thymidine Uptake (cpm)
25000
20000
15000
10000
5000
0
EFNB3-Fc/NHIgG 0 2.5 5 10 (μg/ml)
αCD3 0 0.8 0.8 0.8 (μg/ml)
B
60000
50000
40000
30000
20000
10000
0
NHIgG
EFNB3-Fc
αCD3 1.6 0.8 0.4 0.2 (μg/ml)
EFNB3-Fc/NHIgG 10 10 10 10 (μg/ml)
C
³H-Thymidine Uptake (cpm)
75000
60000
45000
30000
15000
0
αCD3 - + + + -
EFNB3-Fc - + - - +
αCD28 - - + - -
D
30000
25000
20000
15000
10000
5000
0
CD8+ Cells
CD4+ Cells
αCD3 - - + + + + + +
EFNB3-Fc - - + + - - - -
NHIgG - - - - + + - -
αCD28 - - - - - - + +
E
³H-Thymidine Uptake (cpm)
50000
40000
30000
20000
10000
0
PMA + + + + + +
αCD28 + + - - - -
EFNB3 - - + + - -
Inomycin - - - - + +
CsA - + - + - +
F
80000
60000
40000
20000
0
αCD3 (0.8μg/ml) + + + + + + - +
αCD28(μg/ml) 10 5 2.5 - -
EFNB3(10μg/ml) - + - + - + - -
NHIgG (10μg/ml) + - + - + - - -
G
³H-Thymidine Uptake (cpm)
25000
20000
15000
10000
5000
0
Responder
Stimulator
NHIgG
EphA4
EphB3
EphB4
Nil
Mix

Figure 3. Solid phase EFNB3-Fc enhances T-cell proliferation upon TCR stimulation

The affix "anti" used in conjunction with mAbs is simplified as "α" in this and all the other figures of this article. All the concentrations indicated in the figure legends represent those employed during the coating procedure, unless indicated otherwise. Normal human IgG (NHIgG) served as a control for EFNB3-Fc. BALB/c T cells were cultured in wells coated with (A) a suboptimal amount of anti-CD3 (0.8 μg/ml) and different amounts of EFNB3-Fc; (B) a fixed optimal amount of EFNB3-Fc (10 μg/ml) and different amounts of anti-CD3; or (C) a suboptimal amount of anti-CD3 (0.8 μg/ml) along with optimal amounts of anti-CD28 or EFNB3-Fc (both at 10 μg/ml). (D) Magnetic bead-purified spleen CD4 or CD8 cells were cultured in wells coated with suboptimal amount of anti-CD3 (0.8 μg/ml), and optimal amount of anti-CD28 or EFNB3-Fc (both at 10 μg/ml). (E) T cells were cultured in well coated with anti-CD28 (10 μg/ml) or EFNB3 (10 μg/ml); PMA (20 ng/ml), inomycin (1 mg/ml), and CyA (250 nM) were added to the culture solution, as indicated. (F) T cells were cultured in wells coated with anti-CD3 (0.8 μg/ml), EFNB3 (10 μg/ml), and various concentrations of anti-CD28, as indicated. (G) Mitomycin C-treated BALB/c spleen cells ($4x10^5$ cells/200μl/well) were used as stimulators, and C57BL/6 spleen cells ($4x10^5$ cells/200μl/well) were used as responders in MLR; the cells were cultured in the presence of EphA4 (3 μg/ml), EphB3 (3 μg/ml), EphB4 (3 μg/ml), all of the three Ephs (Mix; at 3 μg/ml each), NHIgG (3 μg/ml), or no additional reagents (Nil).

The cells were cultured for 48 h (A-F), or 120 h (G), and their ^{3}H-thymidine uptake in the last 16 h was measured. The experiments were performed more than three times and were reproducible. Means ± SD of triplicate samples from a representative experiment are shown.

Fig. 4

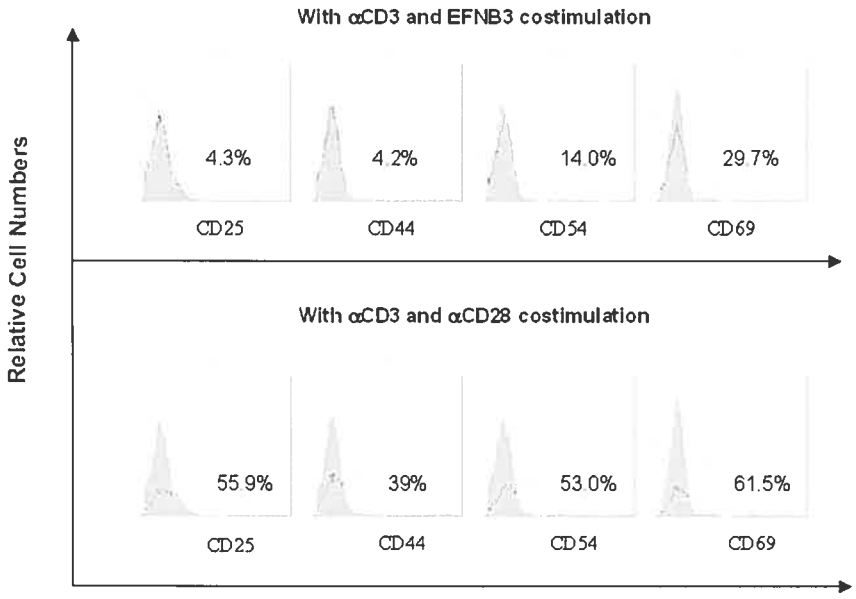

Figure 4. Activation marker expression of EFNB3-Fc-costimulated T cells

BALB/c T cells were cultured in wells coated with anti-CD3 mAb (0.8 μg/ml) plus EFNB3-Fc or anti-CD28 (both at 10 μg/ml). Their activation marker expression at 24 h was assessed by 2-color flow cytometry, using anti-Thy1.2-PE for T-cell gating, and anti-CD25-FITC, anti-CD44-FITC, anti-CD54-FITC, and anti-CD69-FITC for activation markers. Percentages represent activation marker-positive cells among Thy1.2-positive cells in the gated regions after deducting background staining (isotypic Ab controls for mAb against activation markers, shaded area) of the cells. The experiment was performed more than three times and was reproducible. Results from a representative experiment are shown.

Fig. 5

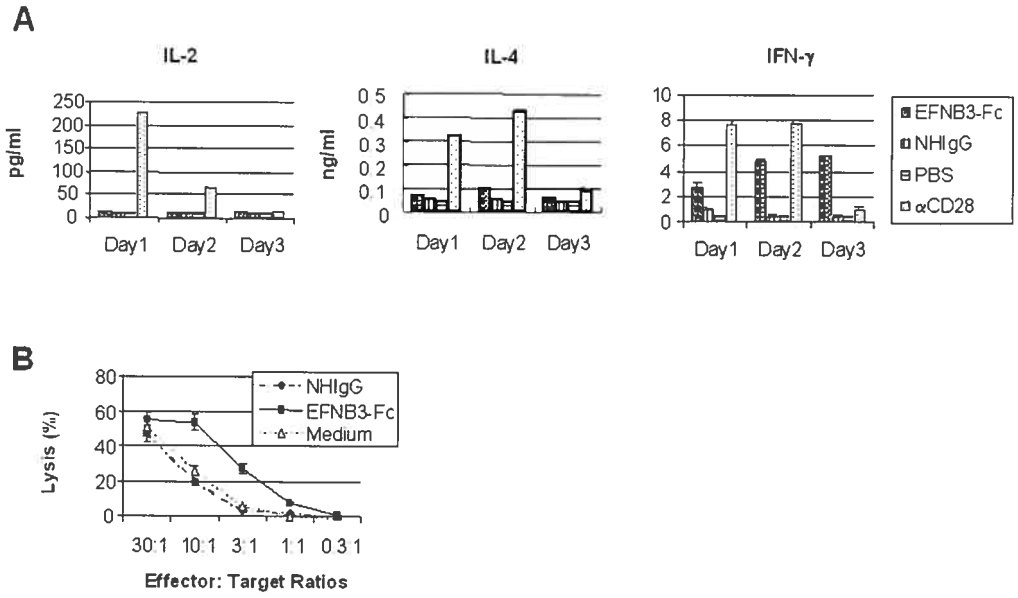

Figure 5. EFNB3-Fc enhances T-cell effector function

A. Solid phase EFNB3-Fc modulates lymphokine production by anti-CD3-stimulated T cells

Mouse spleen T cells were stimulated with solid phase EFNB3-Fc (10 μg/ml) in the presence of a suboptimal concentration of anti-CD3 (0.8 μg/ml). Anti-CD28 and NHIgG (both at 10 μg/ml) were used as positive and negative controls, respectively. Some wells were only coated with anti-CD3, followed by PBS incubation (PBS), and were used as additional blank controls. The culture supernatants were harvested from days 1 to 3, and lymphokines in the supernatants were measured by ELISA. The experiment was performed more than twice and was reproducible. Means ± SD of duplicate samples from a representative experiment are shown.

B. Effect of solid phase EFNB3-Fc on CTL development

2C mouse spleen cells were mixed with an equal amount of mitomycin C-treated BALB/c mouse spleen cells and seeded in flat-bottomed 24-well plates, which were pre-coated with EFNB3-Fc or NHIgG (both at 10 μg g/ml), or not coated (Medium). After 6 days, CTL activity in the stimulated cells was measured by a standard 4-h ^{51}Cr-release assay, using P815 cells as targets. The samples were tested in triplicate, and means ± SD of the percentage of target cell lysis are shown. The experiments were performed twice with similar results, and the data of a representative experiment are shown.

Fig. 6

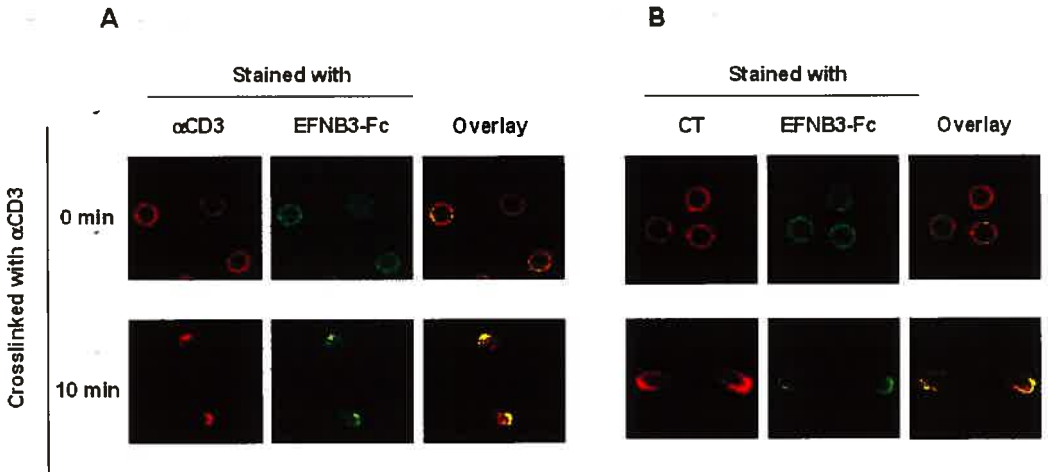

Figure 6. Rapid co-localization of EFNB3R with TCR and raft caps after anti-CD3-crosslinking

BALB/c spleen T cells were crosslinked with anti-CD3 for 0 or 10 min, as indicated. The

locations of TCR (stained with biotin-anti-CD3 followed by Alexa Fluor 594-streptavidin in red) (A), EFNB3R (stained with EFNB3-Fc followed by Alexa 488-anti-human IgG in green) (A and B), and rafts (stained with Alexa Fluor 594–cholera toxin (CT) in red) (B) were revealed by confocal microscopy. All experiments were conducted more than 3 times and were reproducible. Representative results are shown.

Fig. 7

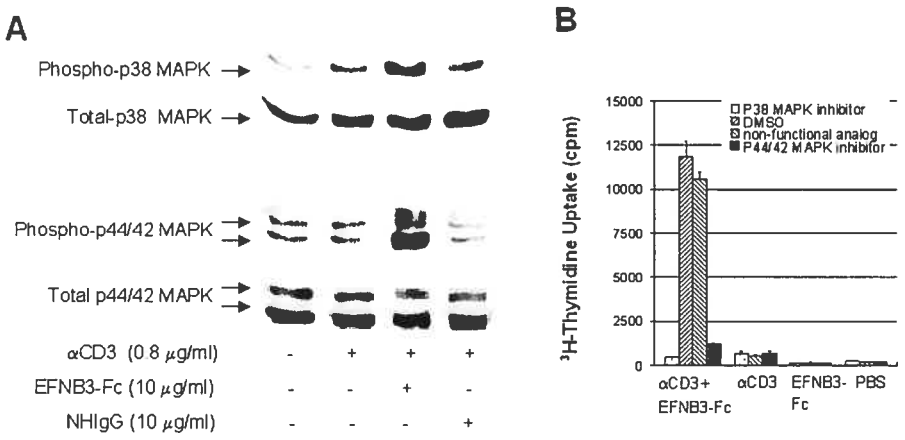

Figure 7. Activation of p38 MAPK and p44/42 MAPK in T cells by solid phase EFNB3-Fc

B. Immunoblotting of p38 and p44/42 MAPK

T cells were added to wells coated with EFNB3-Fc or NHIgG (both at 10 μg/ml) in the presence of a suboptimal amount of anti-CD3 (0.8 μg/ml). The cells were harvested after 2 h, and analyzed by immunoblotting. Arrows indicate signals of p38 phospho-MAPK and total p38 MAPK of the same membrane, and signals of p44/42 phospho-MAPK and total

p44/42 MAPK of the same membrane. The experiment was performed more than twice and was reproducible. Results from a representative experiment are shown.

B. p38 and p44/42 inhibitors specifically inhibit EFNB3-Fc-costimulated T-cell proliferation

T cells were pre-incubated for 1 h in complete culture medium containing the p38 MAPK-specific inhibitor SB203580, the p44/42-specific inhibitor PD98059, and its non-functional structural analogue SB202474 (all at 10 μM), or vehicle (DMSO, dimethyl sulfoxide, 0.1%). The cells were then transferred to wells coated with EFNB3-Fc (10 μg/ml), anti-CD3 mAb OKT3 (0.8 μg/ml), or both, and cultured for 48 h. ^{3}H-thymidine was added to the culture for the last 8 h, and thymidine uptake by the cells was measured. The experiment was performed more than twice and was reproducible. Results from a representative experiment are shown.

Article 3.

The role of ephrinB1 in thymocyte development

Guang Yu, Hongyu Luo, Yulian Wu, and Jiangping Wu

Submitted in Oct. 2004

The role of ephrinB1 in thymocyte development[1]

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Running title: *EphrinB1 regulates T-cell development*

Key words: Eph, ephrinB1, thymocyte, apoptosis, T-cell development

FOOTNOTE

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ABSTRACT

Eph kinases are the largest family of receptor tyrosine kinases, and their ligands, ephrins (EFN), are also cell surface molecules. In this study, we investigated the role of EFNB1 and the Ephs it interacts with (collectively called EFNB1R) in mouse T-cell development. In the thymus, CD8 single positive (SP) and CD4CD8 double positive (DP) cells expressed high levels of EFNB1 and EFNB1R, while CD4 SP cells had moderate expression of both. Soluble EFNB1-Fc in fetal thymus organ culture (FTOC) caused significant subpopulation ratio skew, with increased CD4 and CD8 SP, and decreased DP percentage, while the cellularity of the thymus remained constant. Moreover, in EFNB1-treated FTOC, CD8 SP cells had a significantly enhanced proliferation history, followed by $CD117^{+}$, DP, $CD25^{+}$, and then CD4 SP cells, according to BrdU uptake. In vitro culture of isolated thymocytes revealed that EFNB1-Fc on solid phase protected thymocytes from anti-CD3-induced apoptosis, while EFNB1-Fc in solution augmented such apoptosis. The degree of such protection and enhancing effect on different subpopulations followed the order of their EFNB1R expression levels. At the molecular level, EFNB1R co-migrated with TCR to lipid rafts during TCR activation, and EFNB1R increased TCR signaling strength as evidenced by enhanced phosphorylation of LAT and cbl. These observations collectively suggest the following model: the interaction between endogenous EFNB1 and EFNB1R promotes thymocyte survival, either during positive selection, or in the post-selection period at the SP stage; disruption of such interaction by soluble EFNB1-Fc results in differential degree of apoptosis of according to levels of EFNB1R expression; compensatory proliferation occurred to maintain cellularity homeostasis; the sum of such dynamic changes is the ratio skew of subpopulations. This study reveals that EFNB1 and

EFNB1R are critical in thymocyte development.

INTRODUCTION

Receptors are important cell-surface molecules for communication between cells and their environment. Protein tyrosine kinases (PTK) are essential components in lymphocyte signaling pathways. Thus, receptor tyrosine kinases have dual pivotal functions in lymphocytes. The Eph kinases are the largest receptor PTK family. According to sequence homology, Eph kinases are classified into EphAs (EphA1 to EphA9) and EphBs (EphB1 to EphB6) (1, http://cbweb.med.harvard.edu/eph-nomenclature/cell_letter.html). Ephrins (EFNs), ligands of Eph kinases, are cell surface molecules as well, and can be classified into A and B subfamilies. EFNAs (EFNA1 to EFNA6) are glycosylphosphatidylinositol-anchored proteins, and bind to EphA members with loose specificity; EFNBs (EFNB1 to EFNB3) are transmembrane proteins, and bind to EphBs, again with loose specificity (1). EphA4, an exception, can bind to EFNB2 in addition to EFNA members (2). EFNBs can also function as reciprocal receptors for EphB molecules, and reversely transduce signals into cells (3). Most Eph kinases or EFNs have likely already been identified, because sequences from the human genome project have revealed 14 Eph entries and 8 EFN entries (4).

Since Eph kinases and their ligands are all cell surface molecules, they can only interact with each other when expressed on adjacent cells. Not surprisingly, the clearly demonstrated function of these receptors and ligands is to control accurate spatial

patterning and cell positioning in the central nervous (5, 6) and gastrointestinal (7) systems, and in angiogenesis (8).

Some of the Eph kinases and their ligands are expressed on immune cells (9-12); limited knowledge about their function in immune responses is available, and is described as follows. We have reported previously that a kinase-defective Eph family member, EphB6, is capable of transducing signals into T cells, probably through adaptor molecules associated with its intracellular tail (13). Activation of EphB6 with solid phase anti-EphB6 mAb results in Jurkat cell apoptosis (13), or augmentation of normal human T-cell responses to Ag stimulation (14). EFNB2 and EFNB3, as well as their receptors, are expressed on peripheral T cells. Solid phase EFNB2 and EFNB3 can augment TCR stimulation in vitro (15, 16). Some EphA members have been reported to be expressed on different populations of thymocytes (17), and some EphAs and EFNAs interfere with T-cell development in thymic organ culture (18). EFNA1 can inhibit T-cell chemotaxis (19).

The thymus is the cradle of T cells. Early T-lineage cells in the thymus are usually described as triple negative (TN, $CD3^-CD4^-CD8^-$) cells. Through the rearrangement of TCR, TN cells become immature double positive (DP, $CD4^+CD8^+$) and express a low level of CD3. After this DP transition, the thymocytes undergo extensive positive and negative selections to ensure that the mature T cells are functional and self-tolerant. Positive and negative selection of thymocytes has been reported to occur either in the cortex at the cortex-medullary junction, or within the medulla (20, 21). After selection, the DP cells transiently express high levels of CD3-associated TCR to form mature DP cells, and then

rapidly differentiate into mature CD4 or CD8 single positive (SP) T cells that express high levels of TCR (22).

Positive and negative selection depends on MHC (23,24), on the concentration of peptide presented, and on the accessory molecules involved, all of which are important in determining the strength of TCR signaling (25-27). However, the late stage of positive selection and associated post-selection events seems independent of the same peptide/MHC complexes responsible for the initiation of positive selection (28,29); it is possible that cells adjacent to the T cells under selection express certain cell surface-associated molecules that provide additional signals needed for this stage.

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

MATERIALS AND METHODS

In situ hybridization

A 534-bp cDNA fragment of mouse EFNB1 cDNA from positions 336 to 890 (accession number U12983) was fetched with PCR from a mouse embryonic tissue cDNA library and cloned into pGEM-4Z (Invitrogen, San Diego, CA). The resulting construct, pGEM-4Z-mB1, served to transcribe anti-sense probes with SP6 RNA polymerase or to transcribe sense probes with T7 RNA polymerase, using digoxigenin RNA labeling kits (Roche Diagnostics, Laval, Quebec, Canada). In situ hybridization was carried out according to instructions from the kit manufacturer.

Generation of mouse EFNB-Fc

The coding sequence of the extracellular domains of mouse EFNB1 from positions 255 to 803 was cloned in-frame upstream of the human IgG_1-Fc coding sequence in an expression vector pCMVhFc. The constructs and pcDNA3 were then transfected into CHO/dhfr$^-$ cells with Lipofectamine. The cells were cultured in selection medium (αMEM without ribonucleosides and deoxyribonucleosides containing 5% dialyzed FCS, 0.01 mM methotrexate, 0.8 mg/ml G418 and 0.1 mg/ml gentamycin). After 2 weeks of culture, stably transfected clones were handpicked. Fusion proteins were isolated from supernatants by protein A columns, and verified by N-terminal peptide sequencing (Sheldon Biotechnology Center, McGill University, Montreal, Quebec, Canada). Generation of EFNB2-Fc and EFNB3-Fc has been described previously (15, 16).

Fetal thymus organ culture (FTOC)

Thymuses were sterilely isolated from e17 (embryonic day 17) BALB/c fetuses, and cultured in FTOC medium at 37^0C in 5% CO_2, as described by DeLuca et al. (30). Briefly, the isolated thymic lobes were placed on the surface of a white plain Millipore filter membrane (ISOPORE, 0.4 μM pore size, Bedford, MA), which was supported underneath by surgical gelfoam (ETHICON, Somerville, NJ) in 24-well plates (Costar, Corning, NY). Each well contained 0.8 ml culture medium consisting of Dulbecco's MEM, 20% FCS (Hyclone Laboratories, Logan, UT), streptomycin (100 μg/ml), and penicillin (250 U/ml). Non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (20 nM), and 3.4 mg/ml sodium bicarbonate were added to the medium as supplements. Various recombinant proteins, antibodies or chemical reagents were also included in the medium as indicated in the different experiments. Half of the medium was replaced every

2-3 days until harvesting.

Flow cytometry

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

Confocal microscopy

Five million adult BALB/c thymocytes were reacted on ice for 30 min with 5 μg of EFNB1-Fc and 1 μg each of biotinylated anti-CD3 (clone 2C11, hamster mAb) and anti-CD4. After washing, the cells were incubated with goat anti-hamster IgG (5 μg/sample) for 30 min on ice. The cells were washed with cold PBS and transferred to warm PBS to start the crosslinking process at 37°C. The cells were then immediately fixed with 3.7% formalin. For TCR and EFNB1R staining, respectively, the cells were reacted with streptavidin-Alexa Fluor 594 (1 μg/sample) and goat anti-human IgG-Alexa Fluor 488 (1 μg/10^6 cells) on ice for 30 min. For raft and EFNB1R staining, the procedure was similar as described above, but cholera toxin-Alexa Fluor 594 (0.5 μg/sample) was used in place of streptavidin-Alexa Fluor 594. The Alexa Fluor-conjugated reagents were from Molecular

Probes. The reaction volume of all above staining and cross-linking procedures was always 100 μl. The slides were examined under a confocal microscope.

Immunoblotting

Thymocytes ($10x10^6$ cells/sample) from adult BALB/c mice were incubated with anti-CD3 (clone 2C11, 1μg/ml) and EFNB1-Fc or NHIgG (5 μg/ml) on ice for 1 h, followed by one wash in cold PBS. Anti-hamster IgG and anti-human IgG (both at 1 μg/ml) were then added and the cells were incubated for 30 min on ice; crosslinking (2.5 min) took place by transferring the cells to 37°C. The remainder of the procedure was detailed in our previous publication (31). Briefly, the harvested cells were washed and lysed in lysis buffer for 10 min. For LAT detection, the cleared lysates were resolved in 10% SDS-PAGE with 50 μg protein/lane, and then blotted onto polyvinylidene difluoride membranes; phosphorylated LAT was detected with polyclonal anti-phospho-LAT (Cell Signaling, Mississauga, Ontario, Canada); total LAT was assayed with anti-LAT mAb (Clone 45, BD Transduction Laboratory, Mississauga, Ontario, Canada). For cbl detection, cbl in the lysates (200 μg protein/sample) was first immunoprecipitated with rabbit anti-cbl Ab (Santa Cruz Biotech, Santa Cruz, CA); after 8% SDS-PAGE and blotting, phosphorylated cbl was detected with anti-phosphoprotein mAb (PY20, BD Transduction Laboratory), and total cbl was tested with anti-cbl mAb (Clone 17, BD Transduction Laboratory). Signals were revealed by enhanced chemiluminescence.

RESULTS

EFNB1 and EFNB1R expression in the thymus

Expression of EFNB1 mRNA in the adult thymus was detected by in situ hybridization. As shown in Figure1A, the cortex was highly positive; sparse signals were present in the medulla; the subcapsule region was negative.

Expression of EFNB1 and its receptor(s) on various thymocyte subpopulations at the protein level was then investigated by flow cytometry. The adult and e17 thymus had similar expression patterns, and representative histograms of e17 thymocytes are shown in Figure 1B. For EFNB1 (upper row of Figure 1B), CD8 SP and CD4CD8 DP cells had the highest expression at 29.9% and 29.4%, respectively; the expression on DN (double negative) cells was at a lower level (13.5%); CD4 SP cells had the lowest expression (6.2%). In comparison, another EFNB family member, EFNB3 (upper row of Figure 1B), had low expression in all these subpopulations (3.3% in DN, 1.2% in DP, 2.3% in CD4 SP, and 7.4% in CD8 SP). Such differential expression levels of EFNB1 versus EFNB3 suggest that the former plays a more important role in T-cell development in the thymus.

EFNB1 promiscuously binds to multiple EphB family members (2, 32). The receptor binding of EFNB1 to thymocytes was assessed by EFNB1-Fc staining; such binding revealed the interaction of EFNB1 with the ensemble of all its receptors, which are collectively referred to as EFNB1R, on thymocytes. As shown in the lower row of Figure 1B, EFNBR expression was highest in CD8 SP (40.7%), followed by DP (31.5%) and CD4 SP (8.5%); DN had no detectable expression (0%). In contrast, EFNB3R expression (lower row of Figure 1B), as measured by EFNB3-Fc binding, was low in most subpopulations (0% in DN, 3.2% in DP, and 0.3% in CD4 SP), with the exception of CD8 SP (11.4%), suggesting a more prominent role of EFNB1R than that of EFNB3R in thymic T-cell

development.

The high percentage of DC8 and DP in their expression of EFNB1 and EFNB1R, as revealed in this section, raises an intriguing possibility that these two subpopulations preferentially depend on EFNB1 and EFNB1R for certain developmental processes.

Soluble EFNB1 disturbs subpopulation ratios in FTOC

FTOC was employed to investigate the role of EFNB1 and EFNB1R interaction in thymocyte development in a setting relatively close to a physiological condition; various recombinant proteins were added to the culture. We first verified whether EFNB1-Fc could enter the thymus easily. FTOC was conducted in the presence of EFNB1-Fc (10 μg/ml) for 24h; thymocytes were flushed out, extensively washed, and then reacted with PE-conjugated anti-human IgG; 31.3% of the thymocytes were positive for EFNB1R (right panel, Figure 2A), and this percentage was comparable to that of fresh thymocytes stained with EFNB1-Fc followed by PE-conjugated anti-human IgG (left panel, Figure 2A). This indicates that EFNB1-Fc can effectively enter the FTOC within 24 h.

Next, the effect of EFNBs on FTOC was investigated. After 5-day or 10-day culture in the presence of EFNB1-Fc, EFNB2-Fc, EFNB3-Fc or NHIgG (all at 10 μg/ml), the CD4 and CD8 expression of cells from FTOC was assessed by flow cytometry. As shown in Figure 2B, after 10 -day culture, in comparison with thymocytes from NHIgG-treated FTOC, there was a consistent and obvious decrease of DP (from 69.9% to 37.2%), but an increase of CD4 SP (from 16.0% to 30.1%) and CD8 SP (from 5.4% to 10.9%) percentage in the presence of EFNB1-Fc. On the other hand, EFNB2-Fc and EFNB3-Fc did not drastically

alter the ratios of these subpopulations. Therefore, for the rest of this study, our attention was focused on EFNB1 and EFNB1R; EFNB3-Fc was used for comparison only. At an earlier stage of FTOC, the change of cell population was less drastic than that of day 10, but the tendency was consistent with that of the 10-day culture. For example, on FTOC day 5 (Figure 2C), there was a decrease of DP cell population (from 88.4% to 68.9%) and an increase of CD8 SP cells (from 5.7% to 10.7%) and CD4 SP cells (from 3.2% to 6.3%) in EFNB1-Fc-treated thymus, compared with NHIgG-treated thymus. The effect of another control EFNB3-Fc was similar to NHIgG, except it caused some increase of CD4 SP population. The effect of EFNB3 in this system is not in the scope of this paper.

The ratios of thymocyte subpopulations were determined by a combination of factors, such as proliferation, apoptosis, and extinguishing of CD4 or CD8 expression on DP cells. To gain insight into the reasons for the observed ratio disturbance in EFNB1-Fc-treated FTOC, we measured the proliferation history of each subpopulation by BrdU uptake during the 10-day culture period (Figure 3A). There was a significant increase of BrdU-positive cell percentage in CD8 SP (from 4.4% to 89.7%), CD117 cells (from 31.7% to 63.2%), DN (from 31.7% to 61.3%), CD25 cells (from 17.4% to 53.6%), DP (from 11.3% to 54.5%) and CD4 SP (from 10.2% to 48.0%) subpopulations in EFNB1-Fc-treated FTOC compared with that of NHIgG-treated FTOC. The increase is most significant in CD8 cells. EFNB3-Fc treatment increased the BrdU-positive percentage in these subpopulations to a much less extent. This suggests that in the presence of soluble EFNB1-Fc, thymocytes in FTOC have high rates of turnover.

We next enumerated apoptotic cells on day 10 of FTOC, according to Annexin V staining

(Figure 3B). DP, CD4 SP and CD8 SP cells from FTOC treated with NHIgG had a similar percentage of apoptotic cells, in the range of 13.3% to 20.0%. EFNB1 treatment only moderately increased the percentage of apoptotic cells in DP and CD4 SP cells to 28.5 and 18.8%, respectively, while CD8 SP cells exhibited more apoptosis (43.2%) than other subpopulations. On the other hand, EFNB3-Fc, which was used for comparison, had no apparent effect. It should be noted that the efficiency of apoptotic cell removal in FTOC was not known; such cross-sectional assessment of apoptotic cells on a given day might only reflect the apoptosis in excess of the apoptotic cell removal capacity in the thymus. If we assume apoptotic cells in different subpopulations are eliminated at similar rates, it raises an intriguing possibility that CD8 SP in the presence of soluble EFNB1-Fc undergoes excessive apoptosis; such a high rate has significantly surpassed the removal rate; as a consequence, Annexin V positive cells in the CD8 SP subpopulation has an obvious increase.

Cellularity in FTOC after EFNB1-Fc treatment was investigated after 5, 10 or 12 days of FTOC, and the results (Figure 3C) revealed that the numbers were similar in EFNB1-Fc-, EFNB3-Fc- or NHIgG-treated cultures. Therefore, it appears that a homeostatic mechanism keeps the thymic cellularity constant, despite drastic proliferation and apoptosis in some subpopulations under the influence of soluble EFNB1-Fc

EFNB1R crosslinking promotes thymocyte survival

It is difficult to assess the actual rates of apoptosis of thymocyte subpopulations in FTOC, even if Annexin V positive cells is measured at a given time, because apoptotic cells are constantly removed, and the efficiency of this process cannot be accurately determined. To

understand how soluble EFNB1 caused the ratio skew of different subpopulations, we investigated its effect on isolated e17 fetal thymocytes in the presence of a high dose of solid phase anti-CD3 (10 μg/ml during coating), which enhanced thymocyte apoptosis. Apoptosis of DP, CD4 SP and CD8 SP thymocytes was significantly reduced in the presence of solid phase EFNB1-Fc (top row, Figure 4A), in comparison to that in the presence of solid phase NHIgG (bottom row); interestingly, the magnitude of apoptosis reduction in different subpopulations (CD8 SP, a 45.3% decrease from 47.5% to 2.3%; DP cells, a 23.4% decrease from 39.7% to 16.3%; CD4 SP, a 8.2% decrease from 20.5% to 12.3%) followed the order of their EFNBR1 expression levels, i.e., CD8, DP and then CD4 (from the most to the least decrease of apoptosis; also from the highest to the lowest EFNB1R expression). On the other hand, the effect of EFNB3-Fc on Annexin V expression was much less drastic (middle row, Figure 4A). This suggests that EFNB1R transmits survival signals into these cells. If this is the case, a similar protective effect should occur when EFNB1R on thymocytes receive stimulation from neighboring EFNB1-expressing cells, including other thymocytes; further, blocking such interactions should lead to enhanced apoptosis. This prediction was assessed in a model of isolated e17 fetal thymocytes stimulated with a low dose of solid phase anti-CD3 (2μg/ml during coating) to induce low levels of apoptosis. Soluble NHIgG was added to the culture as a control for EFNBs-Fc to measure apoptosis produced by anti-CD3-induced alone (in the range between 16.4% and 20.5%, bottom row, Figure 4B). When soluble EFNB1-Fc was added to the culture, significant apoptosis occurred in CD8 SP cells (a 32.6% increase from 20.5% to 53.1%), followed by DP (a 10.1% increase from 16.4% to 26.5%), while the degree of CD4 SP apoptosis remained unchanged (a 0.4% increase from 27.1% to 27.5%). It is interesting to note that, again, the degree of apoptosis of each subpopulation was correlated to their

respective EFNB1R expression level. Soluble EFNB3-Fc, which was used for comparison, caused moderately increased apoptosis in CD8 SP and DP, but not CD4 SP cells (second row, Figure 4B; CD8 SP, 12.1% increase from 20.5% to 32.6%; DP, 6.4% increase from 16.4% to 22.8%; CD4 SP, basically unchanged with 1% increase from 27.1% to 28.1%), compared to NHIgG. These results suggest that the exogenous soluble EFNB1-Fc interferes with the anti-apoptotic effect of the endogenous EFNB1 and EFNB1R interaction. The results of this section have raised an intriguing possibility that the observed subpopulation skew in EFNB1-Fc-treated FTOC was driven by excessive apoptosis of CD8 SP and DP cells, as a result of the blockage of the survival signals initiated by endogenous EFNB1.

Signaling events in thymocytes stimulated by EFNB1

To understand the mechanism of the survival signals transmitted via EFNB1R, we first examined EFNB1R and TCR translocation on the cell surface, and the relationship between EFNB1R and membrane lipid rafts immediately after TCR-crosslinking, which was achieved with coating the thymocytes with biotinylated anti-CD3 mAb (hamster IgG) and then crosslinking the mAb with goat anti-hamster IgG. The TCR complex was stained by streptavidin-Alexa Fluor 594 in red, which reacted with biotinylated anti-CD3; EFNB1R, by EFNB1-Fc followed by anti-human IgG-Alexa Fluor 488 in green; the lipid rafts in the T-cell membrane, by cholera toxin-Alexa Fluor 594 in red. Raft, TCR and EFNB1 were evenly distributed throughout the cell surface in resting cells (0 min, Figure 5). After 10-min crosslinking with anti-CD3, TCR rapidly polarized and formed a cap in one end of the cell. EFNB1R also congregated, and co-localized with TCR. Such co-capping lasted more than 20 min (data not shown). Control human IgG (in place of EFNB1-Fc) followed by anti-human IgG-Alexa Fluor 488 detected no signals in these cells (data not shown). After

the CD3 crosslinking, raft also underwent congregation and formed caps, and EFNB1R co-localized with raft caps. Taken together, these data indicate that TCR and EFNB1R both congregate to a raft cap on the cell surface after TCR-crosslinking. This provides a morphological base for EFNB1 to enhance TCR signaling, since now both TCR and EFNB1R are closely associated and located in aggregated rafts, which are scaffolds accommodating many signaling molecules. Perhaps such enhanced TCR signaling provided survival signals to subpopulations of thymocytes, notably DP and CD8 SP cells.

We next examined a couple of signaling molecules involved in T-cell activation. LAT (linker for activation of T cells) is a transmembrane protein, which is phosphorylated after T-cell activation, and its phosphorylation enables it to recruit additional signaling molecules (33). We found that in thymocytes, EFNB1R-crosslinking resulted in augmented LAT phosphorylation over that caused by TCR-crosslinking (Figure 6A), suggesting that EFNB1R could enhance the LAT function. Cbl is an adaptor molecule, and also an E3 ubiquitin ligase; both of these functions are important for T cell activation (34, 35). We demonstrated that tyrosine phosphorylation of cbl after EFNB1R- and CD3-crosslinking was increased compared with CD3 crosslinking alone (Figure 6B), raising the possibility that such phosphorylation allows cbl to interact with SH2 domains of other signaling molecules.

DISCUSSION

In this study, we showed that EFNB1 and EFNB1R were expressed differentially on thymocyte subpopulations, with CD8 and DP cells expressing high levels of both the ligand and its receptors; EFNB1 delivered survival signals via EFNB1R to the thymocytes, and

high EFNB1R-expressing cells seemed to benefit most the effect; FTOC in the presence of soluble EFNB1-Fc had significant subpopulation ratio skew, possibly the result of apoptosis and compensatory proliferation of different subpopulations. At the molecular level, EFNB1R migrated to rafts and co-localized with TCR, when TCR was activated; EFNB1 crosslinking augmented TCR downstream signaling events such as LAT and cbl phosphorylation.

When soluble EFNB1-Fc was present in FTOC, it caused a drastic reduction of DP cells, and an increase of CD4 and CD8 SP cells. The changes were in the percentage of each subpopulation, as the total cellularity remained similar in the EFNB- versus NHIgG-treated thymus. Four findings provide clues to the mechanisms of such changes: 1) accelerated thymocyte proliferation according to BrdU incorporation during the 10-day FTOC in the presence of EFNB1-Fc; 2) increased thymocyte apoptosis according to isolated thymocyte culture in the presence of soluble EFNB1; 3) protection of thymocyte apoptosis by EFNB1 on solid phase according to isolated thymocyte culture; 4) the degree of such apoptosis protection and induction was correlated to EFNB1R expression levels on different subpopulations. Based on these finding, two models could be formulated.

In the first model, soluble EFNB1 enters the thymus, and drives the proliferation of thymocyte subpolulations; such proliferation, in turn, triggers a homeostatic mechanism, which dictates that the total cellularity of a thymus should remain constant; as a result, excess thymocytes were eliminated by apoptosis; the imbalance between proliferation and apoptosis was manifested as altered ratios of subpopulations in FTOC. It is interesting that CD8 SP cells, the subpopulation with the highest EFNB1R expression, has a history of the

most proliferation, while CD4 SP cells with the lowest EFNB1R expression had a history of the least proliferation. Such a correlation can suggest that proliferation is the primary driving force for the observed subpopulations skew in FTOC in the presence of EFNB1. However, we found no evidence that soluble EFNB1-Fc could stimulate isolated thymocytes to proliferate in vitro, in the absence or presence of TCR stimulation (data not shown). The validity of this model is thus very questionable.

In the second model, the soluble EFNB1-Fc causes apoptosis of thymocytes; the consequent loss of thymocytes in FTOC triggers compensatory proliferation to maintain homeostasis; the imbalance of apoptosis and proliferation results in subpopulation ratio skew. In support of this model, solid phase EFNB1 inhibited thymocyte apoptosis in vitro; soluble EFNB1-Fc caused increased apoptosis of isolated thymocytes in vitro, probably due to its interference to the endogenous EFNB1 and EFNB1R interaction between thymocytes; we also found that the subpopulations expressing high EFNB1R on the surface received more benefit of apoptosis protection from solid phase EFNB1, and were more susceptible to apoptosis after the putative EFNB1 and EFNB1R interaction was disrupted by soluble EFNB1-Fc. Overall, this model is more appealing, suggesting that the physiological role of EFNB1 in the thymus is to promote thymocyte survival by delivering anti-apoptotic signals to EFNB1R-bearing cells, notably CD8 SP and DP cells, which had high levels of EFNB1R expression.

How do we explain that CD8 cells, which manifested most extensive EFNB1-Fc-triggered apoptosis, had most significant population increase in FTOC, while DP cells, which were also vulnerable to soluble EFNB-1Fc, showed the sharpest population decrease in FTOC? FTOC is a very complex system, in which proliferation, differentiation, apoptosis, and

apoptotic cell removal occur at the same time; the cell populations we observed after FTOC is the sum of these events. The relative increase of CD8 SP cell could well be that new CD8 SP generation outweighs their apoptosis; the relative decrease of DP population could be due to that their apoptosis outweighs their replenishment. For CD4 SP cells, they are not drastically affected by EFNB1-Fc in terms of apoptosis and proliferation. The increase of their population after FTOC is likely a relative one due to the changes of other populations.

We could not exclude the possibility that in FTOC, soluble EFNB1 promotes extinguishing of CD4 or CD8 expression in DP cells, and this leads to the consequent of SP increase and DP decrease. However, such an effect, even if exists at all, cannot be the major mechanism responsible for skewed subpopulations, because it could not explain the augmented proliferation of most thymocyte subpopulations and increased CD8 SP apoptosis in the presence of soluble EFNB1 in FTOC. Moreover, no rapid conversion of DP to SP cells was found in isolated thymocyte culture in the presence of soluble EFNB1 (data not shown).

Soluble EFNB1-induced apoptosis of isolated thymocytes is presumably due to interruption of the positive interaction between EFNB1 and EFNB1R. As solid phase EFNB1 could protect the thymocytes from apoptosis, which was demonstrated in this study and supported in a previous publication by Roifman's group (36), it is reasonable to speculate that the thymocyte survival depends on signals flowing from endogenous EFNB1 to EFNB1R. In neurons, some EFNBs and EphBs are capable of stimulating each other reciprocally (37-39). Whether EFNB1 on thymocytes can reversely receive signals from EFNB1R, and soluble EFNB1-Fc can cause apoptosis by interrupting such putative reverse signaling are to be further investigated. With that said, we did tested a couple of EphB family molecules

(e.g., EphB6 and EphB4) on solid phase for T-cell survival and proliferation, but no effect was detected (data not shown).

Our study further demonstrated that TCR-crosslinking led to congregation of TCR and EFNB1R into rafts, and EFNBR crosslinking enhanced TCR signaling, as evidenced by augmented LAT and cbl phosphorylation. As thymocytes need to be positively selected based on TCR signaling strength, the physiological role of EFNB1R in highly EFNB1R positive cells, such as DP cells, might be to raise weak TCR signaling to a level over the threshold of positive selection, and thus allow the survival of these cells. The high expression of EFNB1R on CD8 SP cells, and the preferential protective role of solid phase EFNB1 to this population is an interesting observation. Obviously, SP cells have already passed the positive selection period, according to previous reports (40-42). Thus, it seems that EFNB1R might also function as a general trophic factor to enhance the survival of thymocytes. It does not necessarily mean that CD4 SP cells, which do not have high EFNB1R expression, do not need trophic support, but they might depend on other molecules.

We have reported here that solid phase EFNB1 can deliver survival signals through EFNB1R expressed on thymocytes, and blocking EFNB1 and EFNB1R drastically alters the balance of thymocyte populations in FTOC. Due to the inherited limitation of FTOC, in which apoptosis, apoptotic cell removal, putative compensatory cell growth, and phenotype switch could not be individually dissected, we can only use logical reasoning to deduce the role of EFNB1 in T-cell development. To ultimately confirm what we have deduced, i.e., EFNB1 preferentially promotes positive selection and acts as a trophic factor for certain T-

cell subpopulations with high EFNB1R expression, EFNB1 null mutation will be needed. Indeed, we have generated EFNB1 knockout mice, and preliminary results disclosed that these mice were significantly compromised in thymic T-cell development, with atrophic thymus (data not shown).

Through recent studies of ours and several other groups, the important roles of Eph kinases and their ligands, EFNs, in the immune system are emerging; more work in this new dimension of T-cell biology is warranted.

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Fig. 1

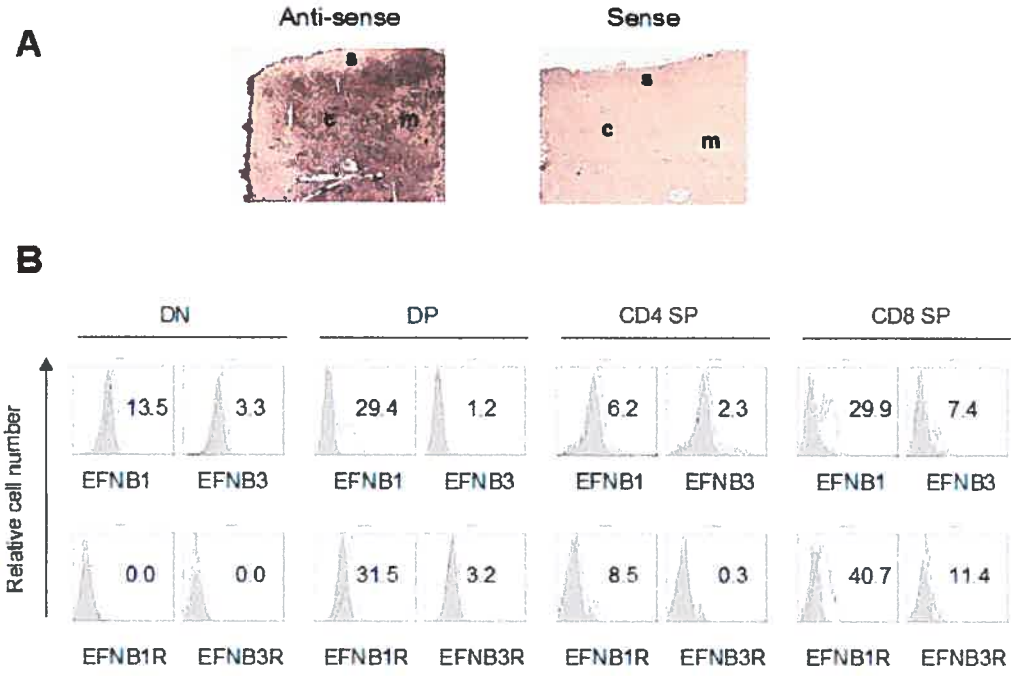

Figure 1. EFNB1 and EFNB1R expression in the thymus

A. In situ hybridization analysis of thymus EFNB1 expression

The adult BALB/c thymus was hybridized with DIG-labeled anti-sense or sense riboprobes of EFNB1. Original magnification is 10x. S: subcapsule region; C, cortex, M: medulla.

B. Flow cytometry analysis of EFNB1 and EFNB1R expression on thymocytes

Three-color flow cytometry was used to assess EFNB1 and EFNB3, or EFNB1R and EFNB3R expression on E17 fetal thymocyte subpopulations. Normal goat IgG and human IgG were served as controls for goat-anti-EFNB Abs and EFNB-Fc, respectively. These controls are shown as shaded areas; such background staining has been deducted from the percentage shown. The experiment was performed more than three times, and representative histograms are depicted.

Fig. 2

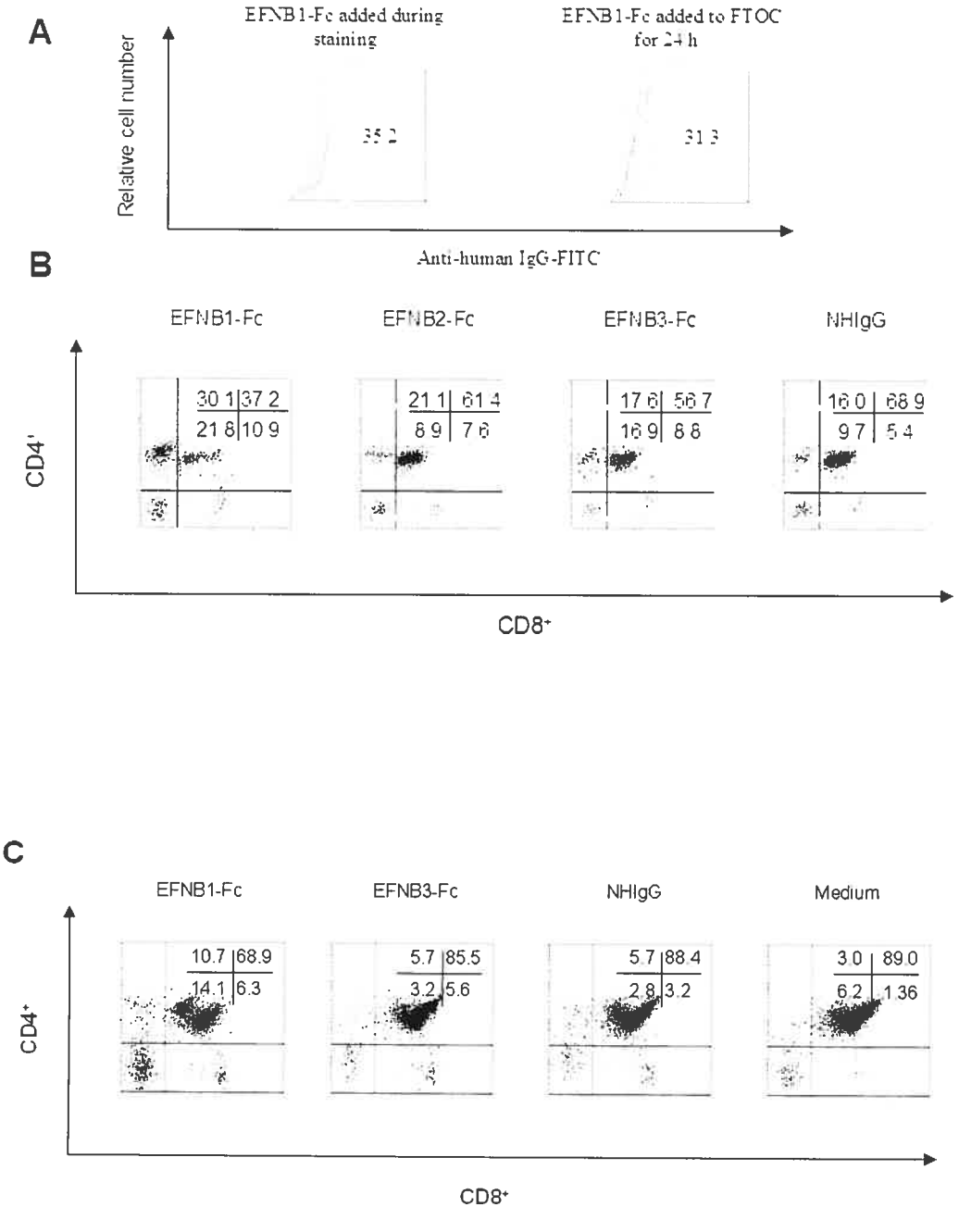
A
EFNB1-Fc added during staining
EFNB1-Fc added to FTOC for 24 h
Relative cell number
35.2
31.3
Anti-human IgG-FITC
B
EFNB1-Fc
EFNB2-Fc
EFNB3-Fc
NHIgG
30.1 37.2
21.8 10.9
21.1 61.4
8.9 7.6
17.6 56.7
16.9 8.8
16.0 68.9
9.7 5.4
CD4+
CD8+
C
EFNB1-Fc
EFNB3-Fc
NHIgG
Medium
10.7 68.9
14.1 6.3
5.7 85.5
3.2 5.6
5.7 88.4
2.8 3.2
3.0 89.0
6.2 1.36
CD4+
CD8+

Figure 2. Soluble EFNB1-Fc skews thymocyte subpopulations in FTOC

Thymuses from e17 BALB/c fetuses were cultured for the durations as indicated. EFNB1-Fc, EFNB2-Fc, EFNB3-Fc or NHIgG (all at 10 μg/ml) was added to the culture. Every 2-3 days, about 50% of the medium was replaced with fresh medium containing the same reagents. The experiment was conducted more than 3 times, and representative data are shown.

A. EFNB1-Fc rapidly enters FTOC

In the left panel, fresh thymocytes from e17 thymus were stained with EFNB1-Fc followed by goat anti-human IgG-PE. In the right panel, EFNB1-Fc was added to e17 FTOC overnight, and the thymocytes was flushed out and stained directly with goat anti-human IgG-PE. NHIgG was used as controls (shaded area). Histograms of 1-color flow cytometry are shown. EFNB1R percentage above the background staining is indicated.

B and C. EFNB1-Fc alters the ratio of thymocyte subpopulations in FTOC

FTOC was conducted for 10 day (B) or 5 days (C) in the presence of EFNB1, 2 or 3, or NHIgG, as indicated. Thymocytes from FTOC were analyzed by 2-color flow cytometry for CD4 and CD8 expression. The percentages of DN, DP, CD4 SP and CD8 SP cells are indicated.

Fig. 3

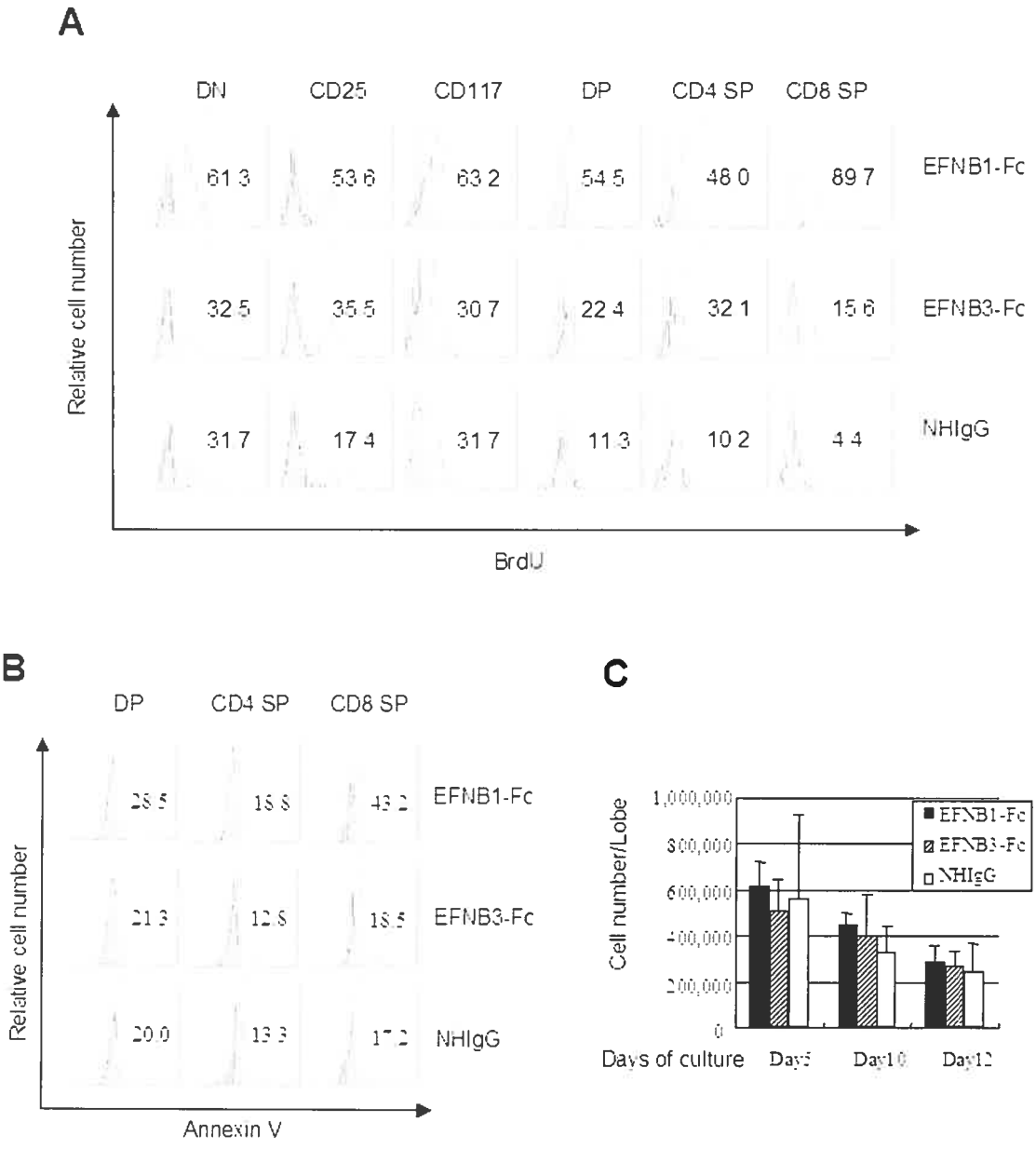

Figure 3. Soluble EFNB1 influences proliferation and apoptosis of thymocytes in FTOC

FTOC of e17 BALB/c thymuses was performed in the presence of BrdU (30 μg/ml) along with various EFNBs and NHIgG as indicated. The experiment was conducted more than 3 times, and representative results are shown.

A. BrdU uptake by thymocytes after 10 days in FTOC

Thymocytes from FTOC without BrdU were stained with FITC-anti-BrdU mAb to establish the background of BrdU staining (shaded area). Two- or three-color flow was employed to assess BrdU uptake of DN, $CD25^+$, $CD117^+$, DP, CD4 SP and CD8 SP cells after 10-day FTOC. Percentage of BrdU positive cells in different subpopulations is shown.

B. Apoptosis of thymocytes after 10 days in FTOC

After 10-day FTOC in the presence of EFNB1-Fc, EFNB3-Fc or HNIgG, thymocytes were reacted with Annexin V-FITC, anti-CD4 and anti-CD8, and analyzed by 3-color flow cytometry. Fresh e17 thymocytes were stained with Annexin V-FITC to determine the background of Annexin V staining (shaded area). Percentages of Annexin V positive cells in different subpopulations are shown.

C. Cellularity of FTOC after different days of culture

After 5, 10 or 12 days of FTOC in the presence of EFNB1-Fc, EFNB3-Fc or NHIgG, thymocytes were flushed out and counted by flow cytometry. The cellularity of each thymus lobe was then calculated. Mean $\pm$ SD of cell number/lobe of 5-10 lobes from 3 independent experiments are illustrated.

Fig. 4

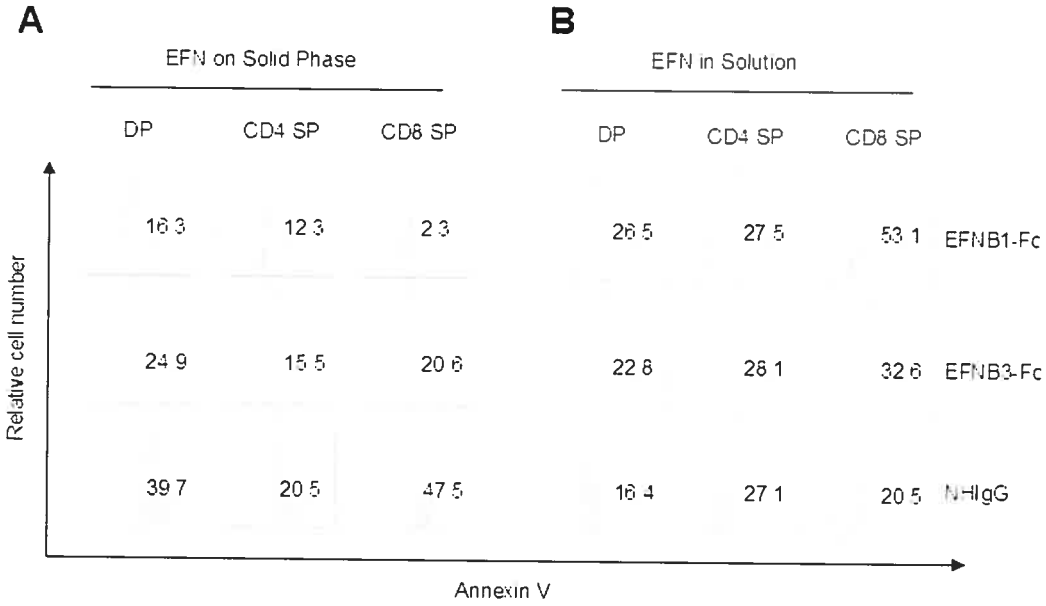

Figure 4. Effect of solid phase and soluble EFNB1-Fc on thymocyte survival in vitro

E17 thymocytes were isolated and cultured in the presence of solid phase anti-CD3 mAb plus solid or soluble phase EFNB1-Fc for 20 h. Apoptosis of different subpopulations was analyzed by 3-color flow cytometry according to Annexin V-FITC staining. NHIgG served as control (shaded area) for EFNB1-Fc; EFNB3-Fc, for comparison. Fresh thymocytes were stained with Annexin V-FITC to determine the Annexin V negative gating. Percentages of Annexin V positive cells are indicated. The experiment was conducted more than 3 times, and representative results are shown.

A. Solid phase EFNB1-Fc inhibits anti-CD3-induced thymocyte apoptosis

Isolated thymocytes were cultured in well coated with high-dose solid phase anti-CD3 mAb (clone 2C11, 20 μg/ml during coating). EFNB1-Fc, EFNB3-Fc or NHIgG was also present during the coating (all at 20 μg/ml). After 20h, different thymocyte subpopulations were analyzed for Annexin V positiveness by flow cytometry.

B. EFNB1-Fc in solution augments thymocyte apoptosis

Isolated thymocytes were cultured in the presence of low-dose solid phase anti-CD3 (clone 2C11, 2 μg/ml for coating), and soluble EFNB1-Fc, EFNB3-Fc or NHIgG (all at 10 μg/ml) for 20 h. Different thymocyte subpopulations were then analyzed for Annexin V binding by flow cytometry.

Fig. 5

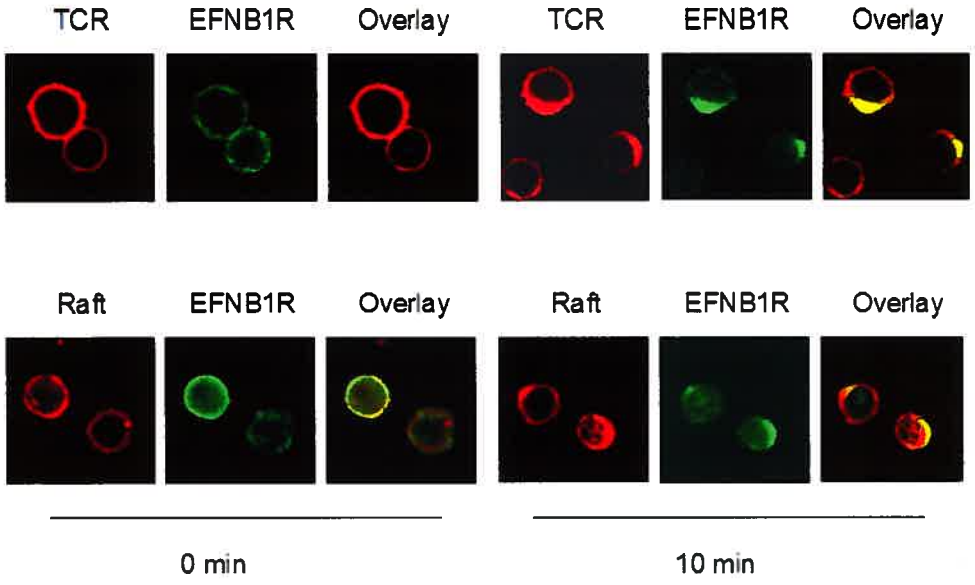

Figure 5. Rapid co-localization of EFNB1R with TCR and raft caps after anti-CD3- and CD4- crosslinking

Adult BALB/c thymocytes were crosslinked with anti-CD3 for 0 or10 min, as indicated. The locations of TCR (stained with biotin-anti-CD3 and biotin-anti-CD4 followed by Alexa Fluor 594-streptavidin in red) (A), EFNB1R (stained with EFNB1-Fc followed by Alexa 488-anti-human IgG in green) (A and B), and rafts (stained with Alexa Fluor 594–cholera

toxin (CT) in red) (B) were revealed by confocal microscopy. The experiment was repeated twice, and a set of representative photos is shown.

Fig. 6

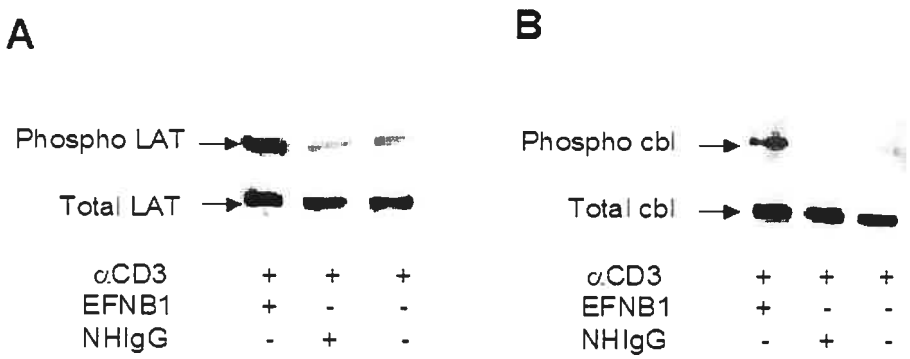

Figure 6. Phosphorylation of LAT and cbl in thymocytes after EFNB1R crosslinking

Adult BALB/c thymocytes were first incubated on ice with anti-CD3 (2C11, hamster mAb), and EFNB1-Fc or NHIgG, as indicated; the cells were then reacted on ice with goat anti-hamster IgG and anti-human IgG; crosslinking was conducted at 37°C for 1.5 min.

A. Phosphorylation of LAT

LAT Phosphorylation was detected with anti-phospho-LAT Ab. The membrane was reprobed with anti-LAT Ab to detect total LAT protein, which indicates similar protein loading in each lane.

B. *Phosphorylation of cbl*

Cbl was immunoprecipitated with anti-cbl Ab. Phosphorylated Cbl was detected by immunoblotting, using anti-phosphoprotein mAb (PY-20). Total cbl protein in each lane was revealed by reprobing the membrane with anti-cbl Ab.

The experiments were repeated at least 3 times, and representative data are shown.

III. DISCUSSION

DISCUSSION

In this project, we studied the functions of EFNBs and of their receptors, EFNBRs, in mouse T cell development and activation. Our data revealed that both ligands and receptors are expressed in peripheral lymphocytes and monocytes/macrophages, with T cells expressing the highest levels of EFNBs and EFNBRs. Solid phase EFNBs-Fc in the presence of suboptimal anti-CD3 crosslinking can enhance T-cell responses in terms of proliferation, activation marker expression, IFN-γ production, and cytotoxic T-cell activity. Upon TCR crosslinking, EFNBRs congregate into aggregated lipid rafts, to which TCR also translocates. This provides a morphological basis for EFNRs to enhance TCR signaling. Downstream from the cell surface TCR, EFNBR signaling augments p38 and p44/42 MAPK activation in T cells. In the thymus, EFNB1 and its receptors have high levels of expression in DP and CD8 SP populations. EFNB1 in solid phase inhibits apoptosis of high EFNB1R-expressing populations, i. e., CD8 SP followed by DP cells. In contrast, soluble EFNB1 augments apoptosis of these cells. These findings suggest that the endogenous EFNB1 and EFNB1R interaction is pivotal for thymocyte survival. When soluble EFNB1 is present in FTOC, there is augmentation of CD4 SP, but a decrease of DP population through a complex homeostatic process.

1. Functional redundancy suggests biological importance of Ephs and EFNs

In vitro, EFNB2 and EFNB3 co-stimulate T cell activation to a similar degree. EFNB1 has

similar effects in this regard (data not shown). These observations are consistent with the highly conserved molecular structures of the three molecules (299, 5), and the overlapping spectrum of their receptors (Fig. 2). The similar expression patterns together with the functional redundancy of these EFNBs suggest the biological importance of EFNBs and of their receptors in maintaining proper T cell function, because such redundancy is the safeguard against lethal consequences due to loss of function of any member. On the other hand, such redundancy represents a challenge to our reductionist approach to analyze the function of these molecules. For example, we have investigated the immune system of EFNB3 knockout mice, but no significant in vitro or in vivo defects could be found (data not shown), suggesting compensation from other members of the family. Simultaneous inactivation of two or more members of the family might be required to reveal their function. Our unpublished data showed that solid phase EFNBs co-stimulate T cell activation, but such an effect could not be blocked by soluble EFNB4, a receptor of EFNBs. The blocking effect of EphB4 was only apparent when EphB6 was mutated, suggesting that EphB4 and EphB6 have functional redundancy in receiving EFNB2 stimulation.

However, different EFNB members do have distinct expression and functional differences. For examples, EFNB1 is more highly expressed on B cells than EFNB2 and EFNB3; soluble EFNB1-Fc but not EFNB2 -Fc or EFNB3-Fc has potent effect on T-cell development in the thymus during fetal thymus organ culture (FTOC). These distinct features allow EFNBs and their receptors to regulate different aspects of T-cell development and activation.

2. Biological significance of EFNBs-EFNBRs in adult T cells

Packaging lymphocytes into defined anatomic locations of secondary lymphoid organs is thought to favor their interactions with antigen-presenting cells and perhaps other regulatory cells, and to provide a framework which permits rapid circulation of naive cells through a space where antigens are concentrated, thus enhancing the efficiency of immune responses. To induce full activation of T cells, co-stimulation is necessary. Co-stimulation molecules play a crucial role in T cell activation (178), proliferation (179), and differentiation to effector cells (180). The co-stimulatory molecules, e.g., CD28 and TNF family, have been well documented. However, besides the co-stimulation provided by the molecules from so called accessory cells, we know little about the other T cell surface molecules that can supply mutual stimulation. One of the fundamental features of co-stimulatory molecules is to reduce the threshold of T cell activation. For example, it has been shown that the presence of CD28 results in a reduction in the threshold number of activated TCR from 8000 to 1500 to achieve T cell activation (300). In vitro studies have demonstrated that in the absence of CD28 engagement, T cells require very high TCR occupancy and prolonged stimulation, whereas CD28 co-stimulation allows T cells to respond to lower degrees of TCR occupancy (300, 301). The T-cell-T-cell collaboration is not a well-investigated topic in immunology, but it has recently drawn certain attention. Wang et al. have demonstrated that the T-cell-surface molecule LIGHT, which is a ligand belonging to the TNF superfamily, can enhance T-cell response via its receptor HveA on T cells in a pure T-cell culture system (262); this is an indication that T-cell-T-cell cooperation is important for optimal T cell response. We have

shown here that both EFNBs and EFNBRs are expressed on T cells, and that solid phase EFNBs can activate purified T cells in the presence of suboptimal levels of anti CD3, suggesting that T cells can receive co-stimulation from fellow T cells, and that EFNBs/EFNBRs are novel pairs of molecules involved in T-cell-T-cell cooperation. It is conceivable that in lymphoid organs, tightly packed T cells constantly interact with each other through EFNBs and EFNBRs; as a consequence, the T-cell response threshold is lowered, as our in vitro data suggested. Naturally, the neighbouring cells could also be other EFNB-expressing cells, e.g., monocytes/macrophages, or B cells. Considering the documented expression of some EphB/EFNB members on endothelial cells (33, 68), it is reasonable to speculate that the interaction between these ligands and receptors have consequences beyond T cell activation. Indeed, EFNB ligands have been found to modify the chemotactic responses of a T cell line and primary T cells (78).

3. Biological significance of EFNB1-EFNB1R in thymocyte development

We have demonstrated that EFNB1 and EFNB1R are predominantly expressed on CD8 SP and DP subpopulations of thymocytes; solid phase EFNB1 can deliver survival signals through EFNB1R expressed on thymocytes, and blocking EFNB1 and EFNB1R drastically alters the balance of thymocyte populations in FTOC.

Thymocytes need to go through extensive selection processes during their development in the thymus. The outcome of the selection is believed to be determined by the strength of interactions between the TCR complex on thymocytes and their ligands on thymic antigen-

presenting cells (157, 302). The current consensus is that thymocytes receiving a moderate-strength TCR signal are positively selected and develop further into mature T cells, whereas cells receiving too weak signals will be eliminated by positive selection, and those receiving too strong signals will commit apoptosis by negative selection.

According to our in vitro data, solid phase EFNB1 inhibited thymocyte apoptosis; on the other hand, soluble EFNB1-Fc caused increased apoptosis of isolated thymocytes, presumably due to its interference to the endogenous EFNB1 and EFNB1R interaction between thymocytes. In addition, compared to the low EFNB1R expressing subpopulations, the thymocyte subpopulations expressing high EFNB1R received more benefit of apoptosis protection from solid phase EFNB1, and were more susceptible to apoptosis after the putative interaction between EFNB1 and EFNB1R was disrupted by soluble EFNB1-Fc. As thymocytes need to be positively selected based on the TCR signaling strength, the physiological role of EFNB1R on highly EFNB1R positive cells, such as DP cells, might be to raise the weak TCR signaling to a level over the threshold of positive selection, and thus allow survival of these cells. Considering that EFNBs can enhance MAPK activation in peripheral T cells (106, 107, 108), and that MAPK activation delivers a major survival signal in CD4CD8 DP cells (302, 303), it seems that EFNB1R may also function to modulate TCR signaling in a manner that prevents induction of apoptosis by low-affinity TCR interactions. However, SP cells have already passed the positive selection period. The high expression of EFNB1R on CD8 SP cells, and the preferential protective role of solid phase EFNB1 with respect to this population is an interesting observation. All these data imply that the

physiological role of EFNB1 in the thymus is to promote thymocyte survival by delivering anti-apoptotic signals to EFNB1R-bearing cells, notably CD8 SP and DP cells, which had high levels of EFNB1R expression. Of course, this does not necessarily mean that CD4 SP cells, which do not have high EFNB1R expression, do not need protection, but they might depend on other molecules for that purpose. It is reasonable to assume that the soluble EFNB1-induced apoptosis of isolated thymocytes is due to interruption of the positive interaction between EFNB1 and EFNB1R.

4. The contribution of this study to science

We have studied the immunological function of EFNBs and EFNBRs interactions, and revealed previously undocumented features of these molecules in T-cell regulation. It is the first extensive study of EFNB members in the immune system in terms of their expression in different immune cell population, function in T cell stimulation, and their effect on T cell development. Our results showed that there is significant functional similarity among the three EFNB members, suggesting that these molecules have overlapping but essential functions in T-cell activation. Our findings led to a new direction in our quest for a better understanding of T cell biology. Furthermore, our study has provided a rationale to use EFNBs and EphBs as therapeutic targets in immune disorders.

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