THE ROLE OF PLEXIN-B2 IN THE IMMUNE SYSTEM

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ABSTRACT

KELLY RONEY: The Role of Plexin-B2 in the Immune System
(Under the direction of Dr. Jenny P.-Y Ting)

Plexins and semaphorins are a family of transmembrane proteins that mediate diverse roles such as cell-cell contact, cell adhesion, cell movement, and cell response. Plexins and semaphorins are unique from other proteins in containing a conserved extracellular semaphorin domain. Semaphorins are smaller than plexins and have short intracellular tails. Plexins are larger and have long intracellular tails that are involved in intracellular signaling. The majority of plexin and semaphorin research has focused on the nervous system since their discovery in the late 1980's. Recently plexins and semaphorins were discovered in the immune system, where they play similar roles similar to those of the nervous system for cell guidance. However, the receptor ligand pairs for semaphorins and plexins in immune system are distinct from those in the nervous system, and plexins and semaphorins have been found to mediate many immune specific cell processes including T cell activation and cytokine response. The B subfamily of plexins has been found to mediate cell movement through activation and deactivation of the Rho family of small GTPases. The majority of B family plexin studies have focused on Plexin-B1 in cell lines or the nervous system. The data in this thesis show that Plexin-B2, and understudied B family plexin, is expressed on cells of the innate immune system *in-vivo*. Plexin-B2 regulates cell velocity, activation of Rho family members Cdc42 and Rac, and cytokine secretion in macrophages and dendritic cells.

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LIST of ABBREVIATIONS

BMDC = bone marrow derived dendritic cells

cDC = conventional dendritic cell

Cdc42 = cell division cycle 42

CFSE = carboxyfluorescein succinimidyl ester

CK2α = casein kinase 2 alpha

c-MET = N-Methyl-N'-nitro-N-nitroso-guanidine) HOS Tranforming gene

DNA = Deoxyribonucleic acid

E. coli = *Escherichia coli*

ERK = extracellular-regulated kinase

G proteins = guanosine nucleotide-binding proteins

GAP = GTPase- activating proteins

GDI = guanine nucleotide dissociation inhibitor

GEF = guanine-nucleotide exchange factor

Grb-2 = growth factor receptor bound protein 2

HER2 = Human Epidermal growth factor Receptor 2

HGFR = hepatocyte growth factor receptor

IPT = an immunoglobulin-like fold shared by plexins and transcription factors

IPT = immunoglobulin like fold, plexins, transcription factor domain

LMWG = low-molecular-weight G proteins

LPS = lipopolysaccharide

MRS = Met related sequence

NCBI = National Center for Biotechnology Information

P3C = Pam3CSK4

PAK = p21-activated kinase

PI(3)K = phosphatidylinositol-3-OH kinase

PLCγ = 1-phosphatidylinositol-4,5-biphosphate phosphodiesterase γ1

PSI = plexins, and semaphorins, and integrin domain

PTEN = phosphatase and tensin homolog

Rac1 = Ras-related C3 botulinum toxin substrate 1

RhoA = Ras homologue gene family member A

Sema3A = Semaphorin 3A

Sema4A = Semaphorin 4A

Sema4D = Semaphorin 4D

SP = Sex and Plexin

Chapter 1: Introduction

1.1 Significance

Plexins and semaphorins are a large family of proteins that are involved in cell movement and response. The importance of plexins and semaphorins has been emphasized by their discovery in many organ systems including the nervous (reviewed in Negishi et al. 2005), epithelial, (Miao et al. 1999; Fujii et al. 2002), and immune systems (reviewed in Kumanogoh et al. 2003; Takegahara et al. 2005) as well diverse cell processes including angiogenesis (reviewed in Klagsbrun et al. 2005), embryogenesis (Perala et al. 2005), and cancer (reviewed in Chedotal et al. 2005). Our lab has identified a novel plexin in the immune system, Plexin-B2, which is expressed on dendritic cells and macrophages and plays a role in the immune response.

Plexins and semaphorins are transmembrane proteins that share a conserved extracellular semaphorin domain of approximately 500 amino acids (reviewed in Gherardi et al. 2004; Kruger et al. 2005). The plexins and semaphorins are divided into eight and four subfamilies respectively based on deoxyribonucleic acid (DNA) sequence similarity. Semaphorins are relatively small proteins containing the semaphorin domain and short intracellular tails, and often serve as the ligand for plexin receptors. Plexins contain the sema domain, as well as the extracellular met related sequence (MRS) domain and long intracellular tails containing a Sex and Plexin (SP) domain (Maestrini et al. 1996; Bork et al. 1999; Tamagnone et al. 1999; Kruger et al. 2005).

The majority of plexin and semaphorin research has focused on the nervous system, particularly the developing nervous system, and most studies are *in-vitro*. Plexins and semaphorins are thought to mediate many common neuronal cell processes including cell movement, dendrite extension and repulsion, cytoskeletal rearrangement, and signal transduction (reviewed in Cohen 2005; Masuda et al. 2005; Negishi et al. 2005). Many neuronal cell processes are similar to those found in cells of the immune system. Both cell types must be able to move within the body in a directional pathway, make and maintain contact with other cells, communicate across cell contact sites, and respond to both intracellular and extracellular cues in order to facilitate these maneuvers. Though historically studied in the nervous system or cell lines, plexins and semaphorins have been recently found to play critical roles in the immune system.

Our lab has historically studied Plexin-A1, which, unlike any other plexins found to date, is under the control of the major histocompatability complex (MHC) class II transactivator (CIITA) and is expressed only in dendritic cells in the immune system (Wong et al. 2003). RNA interference of Plexin-A1 in dendritic cells reduced dendritic cell mediated T cell activation by 90% (Wong et al. 2003). The Plexin-A1 ligand Sema6D is required for CD127 expression, which is critical for late phase T cell development (O'Connor et al. 2008).

Our group has recently identified another plexin, Plexin-B2, on *in-vitro* bone marrow derived dendritic cells (BMDCs) by gene microarray profiling. Originally identified in human brain tumors in 1995 (Shinoura et al. 1995), Plexin-B2 is a 206 kD protein that contains a furin-like proprotein convertase cleavage site found only in the Plexin B subfamily (Tamagnone et al. 1999), suggesting Plexin-B2 is capable of both long and short range

signaling as it can be both membrane bound and secreted. Plexin-B2 is expressed in the developing mouse nervous system (Worzfeld et al. 2004), stimulates neurite growth and induces cell aggregation through trans-homophilic interactions (Hartwig et al. 2005). In transfection experiments with human HEK293T cells, full-length Plexin-B2 is cleaved and heterodimerizes at the cell surface (Artigiani et al. 2003), interacts with the Scatter Factor Receptors Met and Ron (Conrotto et al. 2004), and binds to overexpressed Sema4D (Fig. 1.4) (Masuda et al. 2004).

Our finding of Plexin-B2 gene expression in mouse dendritic cells and macrophages provides evidence of a plexin previously unreported in the innate immune system. We have also found that the expression level of Plexin-B2 is modulated throughout cell maturation, which suggest that Plexin-B2 may play a regulatory role in the life cycle and activation of the dendritic cells and macrophages. We have found the Plexin-B2 is a negative regulatory of cell motility through negative regulation of the small GTPases Rac and Cdc42.

1.3 Plexin and Semaphorin Biology

1.3A. Discovery of Plexins and Semaphorins.

Semaphorins and their receptors, plexins, are a large family of transmembrane proteins that share a conserved semaphorin domain. Semaphorins and plexins are thought to mediate cell guidance and movement through attractive and repulsive cues. A group of proteins that mediate cell movement was first hypothesized by R.W. Sperry in his chemoaffinity hypothesis for nerve fiber growth (1963). This hypothesis was later supported by time lapse video of retinal ganglion growth fibers extending towards the optic tectum in *Xenopus laevis* embryos (Harris et al. 1987). Proteins thought to be involved in guidance

were detected that same year in Xenopus optic tectum by monoclonal antibodies which are today known to recognize Plexin-A1 and neuropilin-1 (Takagi et al. 1987; Ohta et al. 1992; Fujisawa 2004). The first semaphorin was identified as such in 1993 (Luo et al.), and this lead to the discovery that semaphorins are a family of proteins based on homology of a common extracellular semaphorin domain (Luo et al. 1995). The semaphorin family was later expanded to include the semaphorin receptor proteins named plexins, which contain the semaphorin domain as well as an additional intracellular plexin domain (Ohta et al. 1995; Satoda et al. 1995; Maestrini et al. 1996; Comeau et al. 1998). Currently at least 30 semaphorins and nine plexins have been identified.

1.3B. Plexin and Semaphorin Structure

The semaphorin family is divided into eight subclasses based on phylogenetic analysis and protein structure by the Semaphorin Nomenclature Committee (1999). All subclasses contain an approximately 500 amino acid conserved sema domain (reviewed in Kolodkin et al. 1993; Gherardi et al. 2004). Many of the semaphorins also contain a cysteine-rich domain termed plexins, semaphorins, and integrins (PSI) or MRS (Bork et al. 1999; Tamagnone et al. 1999). The first two subclasses, classes I and II, are invertebrate semaphorins. Classes III- VII are vertebrate semaphorins, and class VIII are viral semaphorins. Class III and IV are distinct in that they can be secreted. Class V semaphorins have seven thrombospondin repeats, and class VII semaphorins are glycosylphosphatidlinositol (GPI)-linked to the cell membrane. Class VI semaphorins are prototypical in that they contain only the sema domain, transmembrane domain, and a short cytoplasmic domain.

Like semaphorins, plexins are single pass transmembrane proteins containing an extracellular semaphorin domain, although this domain in plexins is less conserved. Unlike semaphorins, plexins contain three cysteine rich extracellular domains homologous to scatter factor receptors called MRS, an immunoglobulin-like fold shared by plexins and transcription factors (IPT) domain, as well as a long cytoplasmic tail containing an SP domain (Maestrini et al. 1996; Tamagnone et al. 1997; Bork et al. 1999). The plexin domain is a highly conserved 600 amino acid region that shares no homology to any other known domains and mediates signaling through a pathway that has not yet been fully elucidated.

Plexins are found in vertebrates, invertebrates, viruses and bacteria and are divided into four subfamilies based on their DNA sequence (Tamagnone et al. 1999). The plexin family consist of subfamilies A (members A1-A4), B (members B1-B3), C (member C1), and D (member D1) (reviewed in Tamagnone et al. 1999). All subfamilies contain transmembrane proteins, and subfamilies A and B also contain secreted proteins.

The receptor and ligand pairs that mediate semaphorin and plexin signaling have been studied extensively however they vary greatly by cell type, leading to some confusion. Plexins often serve as receptors for semaphorins, and are sometimes coupled with the co-receptor neuropilin (Takahashi et al. 1999; reviewed in Tamagnone et al. 1999; reviewed in Fujisawa 2004). However, the receptor ligand roles are dynamic due to the ability of both plexins and semaphorins to be membrane bound or secreted, transmit signals intracellularly, or bind to other types of proteins and form co-receptors. Semaphorin and plexin roles are further complicated by the ability of these proteins to act as either repulsive or attractive cues, with the type of signal depending on the receptor-ligand pair, developmental stage of the organism, cell type, and/or cellular context (Kantor et al. 2004) .

The majority of semaphorin and plexin research has focused on the nervous system, particularly the developing nervous system. The distal tip of growing neurons is easily studied, very sensitive to small amounts of signal, and thought to grow and extend relative to cytoskeletal rearrangement (reviewed in Goshima et al. 2002). Plexins and semaphorins play both attractive and repulsive roles in neurons, and often lead to cytoskeletal rearrangement thought to be mediated by several different signaling pathways including regulation of the Rho family of GTPases and integrin regulation (Driessens et al. 2001; Oinuma et al. 2004; Tamagnone et al. 2004). Recently, the importance of plexins and semaphorins in basic cell movement and cellular response has been emphasized by their discovery in other organ systems and processes including the epithelial system (Miao et al. 1999; Fujii et al. 2002) , angiogenesis (Serini et al. 2003; Shoji et al. 2003; Conrotto et al. 2005), embryogenesis (Perala et al. 2005), and the immune system (Takamatsu et al. 2010).

1.3C. Plexins and Semaphorins in the Immune System

The immune system and nervous system share many commonalities. Cells in both systems need to be able to move within the body in a directional pathway, make contact and communicate with other cells, and respond to environmental cues. Plexins and semaphorins have been implicated in many of these processes, although the exact role for these proteins and how they signal is not yet fully understood.

Semaphorin 4D (Sema4D), also known as CD100, is the most studied protein of the semaphorins and plexins in the immune system and is a good example of the complex pathways involving these proteins. Sema4D is a 150-kDa cell surface protein that exist as a homodimer on most hematopoietic cells (except red blood cells and platelets) and contains

both a semaphorin domain and a Ig-like domain in its extracellular region (Bougeret et al. 1992; Herold et al. 1995). In T cells, Sema4D associates with CD45 at the cell surface and increases homotypic cell adhesion, while stimulation of CD45 or cellular proteolysis leads to the release of a soluble form of Sema4D (Herold et al. 1996; Elhabazi et al. 2001). There is also evidence that in human T and NK cells, the cytoplasmic domain of Sema4D interacts with a serine-threonine kinase (Elhabazi et al. 1997). Soluble, dimerized Sema4D is capable of inhibiting the spontaneous migration of B cells and monocytes (Delaire et al. 2001). Sema4D expression leads to B-cell aggregation, differentiation, and improved viability *in-vitro* (Hall et al. 1996), and associates with protein tyrosine phosphatase activity (Billard et al. 2000). The receptor for Sema4D in lymphocytes has been identified as CD72 (Kumanogoh et al. 2000), which is in contrast to its receptor in the endothelial and nervous systems, Plexin-B1 (Tamagnone et al. 1999; Conrotto et al. 2005). The binding of Sema4D to CD72 is thought to promote B cell proliferation and differentiation by abrogating the negative effects of CD72 (reviewed in Kumanogoh et al. 2001; Kumanogoh et al. 2004). In Sema4D deficient mice, $CD5^+$ B1 cells are decreased, and *in-vitro* proliferative responses in B cells were reduced compared to wild type mice (Shi et al. 2000). Dendritic cells from Sema4D deficient mice were unable to display co-stimulatory molecules (Kumanogoh et al. 2002).

Other semaphorin and plexin family members have been found in the immune system but their biological roles are only partially understood. Semaphorin 4A (Sema4A) is similar in structure to the above-described Sema4D. Sema4A is found on B cells and dendritic cells and enhances activation of T cells through the receptor Tim-2 (Kumanogoh et al. 2002). Semaphorin 3A (Sema3A) inhibits immune cell migration, similar to the inhibition of

migration by Sema4D (Delaire et al. 2001). Semaphorin 7A (Sema7A), whose function is unknown, is a GPI-anchored protein with high homology to viral semaphorin and is found on activated lymphocytes (Xu et al. 1998; Yamada et al. 1999). Mouse dendritic cells express Plexin-C1. When engaged by its poxvirus semaphorin ligand (A39), Plexin-C1 inhibits cell adhesion, spreading, and migration and induces actin cytoskeleton rearrangement (Walzer et al. 2005). Plexin-A1 is under the control of CIITA and is expressed only in dendritic cells (Wong et al. 2003). Reducing the amount of Plexin-A1 by RNA interference in dendritic cells reduced T cell activation by 90% without affecting antigen binding and presentation (Wong et al. 2003).

Plexin-B2, the focus of this thesis, has been identified but not studied in the immune system. Because the role of Plexin-B2 is not well understood, both Plexin-B2 and its closely related but better-studied family member, Plexin-B1, will be reviewed. Plexin-B1 research has served as the basis for the hypothesis that have guided the research into the immune system function of Plexin-B2. Plexin-B1 is a cell surface protein that mediates many of its functions through Rho family of small G proteins. Therefore G proteins will briefly be reviewed before proceeding to the biology of the B family of plexins.

1.4 G Protein Biology

Guanosine nucleotide-binding proteins (G proteins) are found in cells within every system of the body. G proteins consist of two main families: large and small G proteins. Large G-proteins are composed of α-, β-, and γ subunits and are also called heterotrimeric G proteins. Small G-proteins, also referred to as low-molecular-weight G (LMWG) proteins, consist of only one unit. Both large and small G proteins cycle between an active, GTP-

bound state and an inactive, GDP bound state and serve as molecular switches (Fig. 1.1). G proteins have intrinsic ability to hydrolyze GTP to GDP and are referred to as GTPases, but this reaction rate is generally slow. The rate of GDP/GTP binding is controlled by GTPase-activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). GAPs accelerate the intrinsic GTPase ability of the G protein, and thus downregulate G protein signaling. GEFs serve the opposite function and accelerate the exchange of GDP for GTP and upregulate G protein signaling. Control of G protein signaling can also be regulated spatially by guanine nucleotide dissociation inhibitors (GDIs), which bind and inhibit GTPases by preventing them from binding to the cell membrane, and thus spatially prevent them from interacting with their downstream partners and signaling. Control of G proteins is very finely tuned and very complex. The Rho subfamily alone has to date over 70 GEFs and 60 GAPs to regulate their on/off state, and each of these regulators is in-turn controlled by other proteins (reviewed in Etienne-Manneville et al. 2002; Siderovski et al. 2005; Tybulewicz et al. 2009).

To date, Plexins have been found to interact with only small G proteins. Small G-proteins are a family of proteins containing 50 members, and is referred to as the Ras superfamily. This family is further divided into five subfamilies: 1) Ras, 2) Rho, 3) Rab, 4) Arf, and 5) Ran families (reviewed in Scheele et al. 2007). Of these subfamilies, the B plexins have been found to interact with small G proteins of the Ras and Rho families and the GEFs, GAPs and GDI's that modulate their activation.

1.4A. Ras Subfamily of Small G Proteins

Proteins of the Ras subfamily members includes H-, K-, M-, N-, and R-Ras, Rheb, and Rap. The Ras superfamily must be either extracellular or intracellular membrane linked to function, and have gained considerable attention due to their roles as oncogenes. Ras activating mutations are found in 30% of human cancers, and thus Ras has been targeted for therapeutic intervention (reviewed in Calvo et al. 2010). Ras proteins are capable of translocating between cellular membranes, and Ras localization is thought to be a major method of control of Ras signaling. Similar to other Ras superfamily members, Ras activation can also be controlled by GEFs and GAPs (reviewed in Scheele et al. 2007; Calvo et al. 2010).

Ras is linked to the extracellular-regulated kinase (ERK) signaling pathway and its control of cell proliferation and survival. Mutations in the Ras subfamily are linked to Noonan syndrome, a form of dwarfism with overgrowth of cells, Costello syndrome, in which cells grow in a constitutive manor, and Cardio-facio-cutaneous syndrome, which is characterized by abnormal cell growth (reviewed in Roberts et al. 2007; Lau et al. 2009). Ras mutations have been specifically linked to pancreatic, thyroid, colon, neuronal, liver, skin, and lung cancers with many other types of cancer linked to mutations in the Ras-ERK pathway (reviewed in Roberts et al. 2007). The Ras-ERK pathway has therefore been targeted by many pharmaceutical companies in the treatment of cancer. Ras family proteins have also been linked to T cell receptor and IL-2 receptor stimulation and subsequent cell proliferation and cytokine gene induction (reviewed in Scheele et al. 2007).

1.4B. The Rho Subfamily of GTPases

The Rho subfamily consists of 23 proteins. The members Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42), and Ras homologue gene family member A (RhoA) are the most well studied of the group. This subfamily is distinct in that it contains some atypical members, including Rnd, that are not cycled between the GTP/on and GDP/off for regulation, and are instead thought to be regulated by expression or stability (reviewed in Etienne-Manneville et al. 2002; Tybulewicz et al. 2009). The Rho family of GTPases mediates cell shape, migration, and adhesion as well as other cell processes including cell activation, proliferation, and survival. For example, in hematopoietic stem and progenitor cells (HSC/Ps), Cdc42 and Rac deletional mutants show a loss of proliferation and increased apoptosis, as well as defects in adhesion, migration, and homing capabilities. T and B cells deficient in Cdc42 or Rac also display defects in proliferation, survival, migration, and adhesion (reviewed in Mulloy et al. 2010). In macrophages, Rac and Cdc42 are implicated in Fcγ receptor-mediated phagocytosis as well as migration, cell shape, and adhesion (reviewed in Ridley 2008; Mulloy et al. 2010). Rac, Rho, Rnd, and Ras, as well as other GTPases, have been linked to B family plexins.

1.5 The Biology of B Family Plexins

The B family plexins are one of the four main plexin families, A-D. The B plexin family is composed of three members, Plexin-B1, B2, and B3 (Fig. 1.2). The B family plexins show the highest homology to the scatter factor receptor family, which is a family of transmembrane receptors that lead to invasive growth and are often linked to cancer (Maestrini et al. 1996; Conrotto et al. 2004). All Plexin-B family members share the protein domains common to all plexins, including the extracellular semaphorin and MRS [also called

plexins, and semaphorins, and integrins (PSI)] domains, and the three intracellular immunoglobulin like fold, plexins, transcription factors (IPT) domains, followed by the Plexin Cytoplasmic RasGAP domain (also known as SP domain). Plexin-B1 and B2 have a distinct protein domain that separates them from the other plexins, the furin-like protease domain, which are thought to facilitate cleavage by proteases (Tamagnone et al. 1999).

Plexin-B1 was the first of this family identified, and has been the most extensively studied, particularly in its role as a regulator of the Rho family of small GTPases. Thus, Plexin-B1 is a model B family plexin from which hypothesis can be generated for other B family plexins. A critical review of Plexin-B1 biology and roles in the immune system allows for comparisons between Plexin-B1 and Plexin-B2 and highlights unanswered questions for Plexin-B2 and the B family of plexins.

1.6 Plexin-B1 Biology

1.6A. Plexin-B1 and RacGTPase

In Drosophila, which has only one B family plexin, Plexin-B has been found to bind and inhibit only active, GTP-bound Rac and enhance RhoA signaling at the same time, suggesting that Plexin-B in this system functions to fine tune the Rho family GTPase signaling (Hu et al. 2001). The binding of Plexin-B1 to Rac-GTP but not Rac-GDP to has been found for Plexin-B1 and B2 in vertebrates, and thus this function of the B family plexins is conserved across species (Fig. 1.3) (Rohm et al. 2000; Vikis et al. 2000; Driessens et al. 2001). Plexin-B1 binds to activated Rac via a sequence that is partially homologous to the CRIB (Cdc42/Rac interactive binding) domain in the Plexin-B1 cytoplasmic tail (Vikis et

al. 2000; Driessens et al. 2001). This domain binds to the switch I region (effector domain) of Rac (Vikis et al. 2000).

The function of Plexin-B1 binding to active Rac is poorly understood. In Drosophila Plexin-B, dosage assays suggest that Plexin-B inactivates Rac, yet how this occurs has not yet been determined (Hu et al. 2001). In HEK293T cells Plexin-B1 inhibition of Rac signaling is accomplished by binding of activated Rac to Plexin-B1 and sequestering it away from its downstream effector p21-activated kinase (PAK), though the phenotypic consequence of Plexin-B1 Rac sequestration from PAK is not reported (Figure 3) (Vikis et al. 2002). It has also been suggested that Plexin-B1 could function directly as a Rac GEF (Rohm et al. 2000; Hu et al. 2001; Vikis et al. 2002). However, extensive structural studies of Plexin-B1 have suggested that it does not function as a RacGAP (Bouguet-Bonnet et al. 2008). Bouguet-Bonnet et al. have demonstrated that the GTP binding state of Rac does not change in response to titration of Plexin-B1, and thus the role of Plexin-B1 is likely that of an effector of Rac but not a direct GAP (2008). These structural studies have also suggested that Rac binds to only undimerized Plexin-B1, and that the Rac-Plexin-B1 interaction is temperature and pH dependant (Hota et al. 2009; Tong et al. 2009). Human prostate cancer clinical samples have been found to have mutations in Plexin-B1 Rac binding site (Wong et al. 2007). These mutations, when cloned and overexpressed in HEK293T cells, upregulate active Rac and increased cell motility, invasion, and adhesion (Wong et al. 2007). Together these data suggest that regulation of Plexin-B1, and regulation of the small GTPases by Plexin-B1, is very finely tuned by temperature, pH, dimerization state of Plexin-B1 by its ligand, and cell type.

Interestingly the binding of activated Rac to Plexin-B1 enhances the amount of Plexin-B1 and its localization to the cell surface, suggesting bidirectional signaling, and is, according to the authors, the first known example of a small GTPase directly regulating the function of a receptor (Vikis et al. 2000; Vikis et al. 2002). Biochemical experiments using one of the ligands for Plexin-B1, Sema4D, have shown that ligand binding to Plexin-B1 enhances the interaction of Plexin-B1 and Rac (Vikis et al. 2000).

Plexin-B1 binding to activate Rac has been confirmed in many papers and across species, suggesting that this is likely an important function of Plexin-B1 and other B family plexins. The Plexin-B1-Rac interaction has been shown to function to downregulate Rac, although the mechanism is unknown. Future studies should explore how this downregulation occurs, what ligand binding state or dimerization state of Plexin-B1 is required for this downregulation, and determine the phenotypic consequences.

1.6B. Plexin-B1 and its Effects on Rho Activation

Unlike Rac, which is bound by Plexin-B1 only in its active, GTP-bound state, both RhoA-GDP and RhoA-GTP interact with Drosophila Plexin-B (Hu et al. 2001). Studies in mammalian and yeast cells lines have shown that Plexin-B1, B2 and B3 but not A2 and D1 bind to Rho guanine nucleotide exchange factors (RhoGEFs) containing a post synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein (PDZ) domain instead of directly to Rho (Ranganathan et al. 1997; Hu et al. 2001; Driessens et al. 2002). The Plexin-B family control of Rho is mediated through dimerization of the PDZ domain found in the Plexin-B cytoplasmic tail with the PDZ domain of the RhoGEFs PDZ-RhoGEF and Leukemia-associated RhoGEF (LARG) (Hu et al. 2001). PDZ

dimerization results in cell rounding and retraction and the formation of stress fibers in overexpression studies in Swiss3T3 fibroblast and in HEK293T cells (Hu et al. 2001; Aurandt et al. 2002; Driessens et al. 2002). The binding of Plexin-B1 to PDZ-RhoGEF and LARG in primary hippocampal neurons and retinal ganglion leads to growth cone collapse in response to Sema4D (Swiercz et al. 2002). In COS-7 cells it has been shown that a direct interaction between Plexin-B1 and another Rho family member, Rnd1, amplifies the interaction of Plexin-B1 and Rho, and thus Plexin-B1 is modulated by a Rho family member (Oinuma et al. 2003). *In-vivo* experiments in using *Plxnb1*$^{-/-}$ mice demonstrate that long term, Sema4D induces dendrite branching in hippocampal neurons post the immediate effect of growth cone collapse (Vodrazka et al. 2009).

Intriguingly, Plexin-B1 interacts with p190RhoGAP in a ligand dependant manner and can thus downregulate Rho (Barberis et al. 2005). This suggests that Plexin-B1 is well positioned and equipped to respond to environmental cues and down- or up-regulate Rho as needed.

Plexin-B1 Rac and Rho binding are separate events. Studies with mutated Plexin-B1 that does not contain the Rac binding domain show that it can dimerize and activate Rho normally (Driessens et al. 2001). This finding is supported by structural studies which suggest that Rac1 binding is not compatible with Plexin-B1 dimer formation, and thus ligand-induced Rho activation similar to wild type Plexin-B1 (Hota et al. 2009). Additional studies of overexpressed Plexin-B1 in 3T3 cells suggest that undimerized Plexin-B1 does not affect cell morphology, and that clustering of the cytoplasmic domain of Plexin-B1 by ligand is required for cell contraction and stress fiber formation (Driessens et al. 2001).

1.6C. Plexin-B1 and Ras GAP Activity

In a pivotal 2004 paper, Plexin-B1 was shown to have yet another function in modulating the Rho family of GTPases: Ras-GAP (Oinuma et al.). Previous studies had noted homology between the intracellular domains of Plexin-B1 and Ras-GAPs at two conserved arginine residues (Rohm et al. 2000; Hu et al. 2001). Oinuma et al. found that Plexin-B1 directly interacts with the active, GTP bound from of the Rho family member R-Ras (2004). This interaction depends on the binding of Plexin-B1 to the Rho family member Rnd1 and results in the activation of the intrinsic GTPase ability of R-Ras in a ligand dependant manner in both overexpression studies with COS-7 cells and studies of endogenous Plexin-B1 in PC12 cells (neuroendocrine tumor cell line) (Oinuma et al. 2004). The GAP activity of Plexin-B1 towards R-Ras induces neurite retraction and inhibits extracellular matrix-mediated cell migration in response to Sema4D, and functions independently of the modulation of Rho by Plexin-B1 (Oinuma et al. 2004; Oinuma et al. 2006). Plexin-B1 has also been shown to be a GAP for M-Ras in COS-7 cells and neurons (Saito et al. 2009). The interactions between Plexin-B1 and R- and M-Ras affect the downstream signaling of R and M-Ras, including suppressing phosphatidylinositol-3-OH kinase [PI(3)K] signaling and activation of phosphatase and tensin homolog (PTEN) (Oinuma et al. 2006; Saito et al. 2009; Oinuma et al. 2010). PI(3)K and PTEN are major regulators of PIP_3, a key modulator of many cell processes including cell growth, migration, and survival that is often implicated in cancer and autoimmunity (Zhang et al.; Patel et al. 2005).

1.6D. Plexin-B1 and c-Met

Plexin-B1 is tyrosine phosphorylated within minutes of binding to Sema4D in porcine endothelial cells (Basile et al. 2005). This phosphorylation of Plexin-B1 is required for activation of RhoA by Plexin-B1 and is meditated by epidermal growth factor receptor B-2 (ErbB-2, also known as Human Epidermal growth factor Receptor 2 [HER2] in humans and neu in rodents) and N-Methyl-N'-nitro-N-nitroso-guanidine HOS Tranforming gene (c-Met) (Swiercz et al. 2002; Swiercz et al. 2004; Swiercz et al. 2008; Swiercz et al. 2009). ErB-2 is a member of the family of epidermal growth factor receptor tyrosine kinases. c-Met, also called hepatocyte growth factor receptor (HGFR), is a member of the scatter factor receptor family. c-Met associates in a complex with Plexin-B1 in a ligand independent manor and is itself phosphorylated by ligand binding to Plexin-B1 (Giordano et al. 2002). This co-stimulation leads to the ability of cells to migrate towards Sema4D and to invasive growth in liver progenitor cells (Artigiani et al. 2003). The interaction of c-Met and Plexin-B1 requires the extracellular domain of both proteins (Giordano et al. 2002). The interaction between plexin and c-Met is mediated exclusively by the B family plexins and not other plexins (Conrotto et al. 2004). Surprisingly in metastatic melanoma cells Plexin-B1 is diminished compared to benign melanomas, and expression of Plexin-B1 suppresses activation of c-Met and migration in response to hepatocytic growth factor (Stevens et al. 2010). However Plexin-B1 did lead to the activation of Akt in melanoma cells, suggesting that Plexin-B1 may function in both tumor suppression and progression (Stevens et al. 2010). The phosphorylation of Plexin-B1 is not required for its ability to bind to Rac-GTP or function as an R-Ras GAP (Swiercz et al. 2009).

1.6E. Plexin-B1 Binds to PLCγ

Swiercz et al. has shown that Plexin-B1 binds to both 1-phosphatidylinositol-4,5-biphosphate phosphodiesterase γ1 (PLCγ) and growth factor receptor bound protein 2 (Grb-2) upon activation by Sema4D in an Erb-2 containing breast cell line (Swiercz et al. 2009). This binding is not observed in the breast cell line MDA-MB-468, which does not express Erb-2 (Swiercz et al. 2009). PLCγ1 binding to Plexin-B1 is dependent on the tyrosine phosphorylation of Plexin-B1 by Erb-2 to create a docking site for PLCγ (Swiercz et al. 2009). PLCγ binding to Plexin-B1 results in activation of PDZ-Rho GEF through the SH3 domain of PLCγ and induction of migration towards Sema4D and growth cone collapse in hippocampal neurons (Swiercz et al. 2009). In ErbB-2 deficient cells, migration towards Sema4D but not fetal bovine serum is abolished, suggesting that PLCγ1 is required for Sema4D specific migration but not all migration (Swiercz et al. 2009). This pivotal research links the activation of Plexin-B1 by its ligand Sema4D to its function as an activator for PDZ-RhoGEF and subsequent activation of Rho through creation of a docking site for PLCγ via phosphorylation of Plexin-B1 by ErbB-2. This data also demonstrates that the processes involved in the Rho activation signaling pathway are separate from Plexin-B1 Rac-GTP binding and Plexin-B1's function as a RasGAP as these two processes are not affected by mutations to Plexin-B1 that render it unable to be tyrosine phosphorylated by Erb-B2 (Swiercz et al. 2008; Swiercz et al. 2009). Additionally, c-Met phosphorylates Plexin-B1 in a different location than ErbB-2, and thus c-Met induces different downstream effects than Erb-B2, including pro and anti-migratory effects (Swiercz et al. 2008; Swiercz et al. 2009).

1.6F. Summary Model of Plexin-B1 Signaling

Plexin-B1 signaling is very complex (Fig. 1.3). One way to simplify the diverse functions of Plexin-B1 is to separate the known signaling components that function independently from those that function in response to ligand. Ligand independent functions of Plexin-B1 include binding to c-Met and RacGTP. c-Met can be phosphorylated by either its ligand HGF or by Plexin-B1 binding to its ligand Sema4D (Giordano et al. 2002). However, recent studies have demonstrated that c-Met is not phosphorylated in response to Sema4D, and c-Met phosphorylation is suppressed by expression of Plexin-B1 in melanoma cells (Stevens et al. 2010). Plexin-B1 has also been shown to interact with active, GTP bound Rac, leading to sequestration of Rac from its downstream effector PAK (Vikis et al. 2002). The interaction of Plexin-B1 and active Rac has been characterized structurally and is thought to change the Plexin-B1 structure so that it is no longer able to dimerize (Hota et al. 2009; Tong et al. 2009). It is possible that binding to Rac changes Plexin-B1 so that it is no longer able to be dimerized in response to ligand, and thus can not activate RhoA. However, studies in Drosophila suggest that Plexin-B downregulates Rac while enhancing RhoA activity (Hu et al. 2001). Rac binding to Plexin-B1 is greatly increased by Sema4D binding to Plexin-B1 (Vikis et al. 2000), suggesting crosstalk between the two signaling pathways.

Upon binding its ligand Sema4D, Plexin-B1 activates Rho through binding of its PDZ domain to the PDZ domain of the RhoGEFs PDZ and LARG (Aurandt et al. 2002; Driessens et al. 2002). PDZ and LARG increase the exchange of GDP to GTP on Rho, increasing the amount of the active, GTP-bound form of Rho in the cell. Plexin-B1 also acts as a GAP for R-Ras in response to its ligand, Sema4D (Oinuma et al. 2004). The Rho GTPase Rnd1 is required for Plexin-B1 to function as an R-RasGAP (Oinuma et al. 2004). Plexin-B1, upon Sema4D activation, shuts down R-Ras activation that occurs in response to adhesion to

fibronectin, and this results in suppression of R-Ras mediated activation of PI(3)K, inhibition of adhesion dependant cell migration and growth cone collapse (Oinuma et al. 2006). Plexin-B1 mediates the suppression of PI(3)K pathway through dephosphorylation and activation of PTEN, which functions as a phosphatase for PIP_3 and therefore downregulates PI(3)K/PIP_3 pathway signaling (Oinuma et al. 2010). The upstream pathway of Plexin-B1 mediated downregulation of PTEN is unknown, but is thought to involve casein kinase 2 alpha (CK2α), which phosphorylates and inactivates PTEN and is downregulated by Sema4D (Oinuma et al. 2010). Plexin-B1 also serves as a GAP for M-Ras, which leads to downregulation of ERK activity and reduced dendrite outgrowth in cortical neurons (Saito et al. 2009).

Another group found that Sema4D binding to Plexin-B1 upregulates PI(3)K, AKT, ERK and migration (Basile et al. 2005; Aurandt et al. 2006). How is this possible? These dual functions are thought to be mediated by the binding state of Plexin-B1 to the receptor tyrosine kinases c-Met and ErbB-2 (Swiercz et al. 2008). Plexin-B1 is preferentially bound to c-Met, and under these conditions RhoA and resulting migration is inhibited (Swiercz et al. 2008). Plexin-B1 can also be found in complex with and be phosphorylated with ErbB-2, and this mediates activation of RhoA and migration through creation of a PLCγ docking site on Plexin-B1 for RhoGEFs (Swiercz et al. 2008; Swiercz et al. 2009). c-Met and ErbB-2 are found alone or together in some cell types, and control of Plexin-B1 through these two receptor tyrosine kinases may be mediated by expression of c-Met and ErbB-2 or competitive binding. Additionally, the Rho-GTPase p190 associates transiently with Plexin-B1 upon ligand stimulation and downregulates Rho (Barberis et al. 2005).

How the activity of Plexin-B1 as an R-RasGAP, binding partner to ErbB-2, c-Met, RacGTP, and p190 fits together as a signaling pathway has yet to be elucidated, and will likely require cell specific studies as well as studies of specific sites within cells to fully understand this complex signaling cascade. The regulation of Plexin-B1 signaling by ligand binding and/or the presence of other plexins and semaphorins within the same cell remain pivotal questions critical to fully understanding Plexin-B1 biology.

1.6G. Plexin-B1 in the Immune System

Plexin-B1 is found on immature bone marrow derived cDCs but not mature cDCs, and is absent on monocytes (Chabbert-de Ponnat et al. 2005). In immature cDCs, soluble Sema4D, a ligand for Plexin-B1, inhibits migration, and this inhibition can be blocked by antibody against Plexin-B1 (Chabbert-de Ponnat et al. 2005). Plexin-B1 is also expressed on bone marrow stromal cells, activated T cells, and follicular dendritic cells, and T cells and upon encounter with Sema4D on B cells leads to increased B cell proliferation (Granziero et al. 2003). This limited exploration of Plexin-B1 in the immune system reflects a paucity of research in this area.

1.7 Plexin-B2 Biology

Plexin-B2 is much less studied than the closely related Plexin-B1. Studies of the phenotype of the *Plxnb2*$^{-/-}$ mouse in the nervous system reveal that Plexin-B2 is required for proper cell homing and neural tube closure (Friedel et al. 2007) The majority of data published for Plexin-B2 stems from Plexin-B1 focused articles in which Plexin-B2 was also included in limited experiments. To date Plexin-B1 and Plexin-B2 share similarities in their

interactions with Rho family GTPases despite the fact that the intracellular tails of Plexin-B1 and B2 are only 61% identical at the protein level (data not shown). Plexin-B2 considered in the context of Plexin-B1 allows for immediate hypothesis and research direction (Fig. 1.4).

1.7A. Plexin-B2 and RacGTPase

The Rho family small GTPase Rac has been implicated in many cell functions, including cell migration, adhesion, and morphology. Similar to Plexin-B1, Plexin-B2 overexpressed in *Escherichia coli* (E. coli) has been shown to bind to active, GTP bound form of Rac (Driessens et al. 2001). Unlike Plexin-B1, in which many studies in mammalian cells have concluded that Plexin-B1 binds to Rac, the Plexin-B2 Rac interaction has not been studied in mammalian systems. We can hypothesize that Plexin-B2 will also bind to Rac in mammalian cells, and will mediate functions similar to those found for Plexin-B1 including cell migration, adhesion, and morphology.

1.7B. Plexin-B2 and its Effects on Rho Activation

Plexin-B2, similar to Plexin-B1, can associate with the Rho GEFs PDZ and LARG in yeast two hybrid screens and overexpression studies in HEK293 T cells (Driessens et al. 2002; Perrot et al. 2002; Swiercz et al. 2002). When Plexin-B2 is overexpressed in Swiss-3T3 fibroblast cells and synthetically dimerized by replacement of extracellular Plexin-B2 with TrkA, the receptor for nerve growth factor, Plexin-B2 upregulates active Rho through binding of the PDZ domain found in the cytoplasmic tail of Plexin-B2 to the PDZ domain of PDZ and LARG RhoGEFs (Perrot et al. 2002). The RhoGEFs then exchange the inactive GDP-bound form of Rho to the active GTP-bound form of Rho (Perrot et al. 2002). The

activation of Rho by dimerized Plexin-B2 in Swiss-3T3s induced stress fibers, which are characteristic of Rho activation (Perrot et al. 2002). The binding of the B family plexins to PDZ-Rho and LARG is specific, as both Plexin-B1 and B2 were shown to not interact with the Rac-GEF Tiam 1, or the Rho-GEF p115RhoGEF, which does not contain the PDZ domain (Perrot et al. 2002). Additionally, the activation of Rho requires dimerization of Plexin-B2, which is opposite of the interaction of Plexin-B1 and B2 with Rac (Perrot et al. 2002).

The observation that Plexin-B2 interacts with Rho through PDZ and LARG suggests that the Plexin-B2 modulation of Rho is identical to the modulation of Rho by Plexin-B1. The interaction with PDZ-Rho and LARG has also been shown in Drosophila Plexin-B, suggesting that this mechanism of control of Rho is highly conserved (Hu et al. 2001) and is likely an important function of B family plexins.

1.7C. Plexin-B2 and RasGAP Activity

Plexin-B2 has not been directly studied for its effects as a RasGAP. One study has shown that in COS-7 cells, when any of the B family plexins are expressed with Rnd1 and then stimulated by Sema4A, cells display growth cone collapse, a phenotype associated with downregulation of R-Ras by Plexin-B1 (Yukawa et al. 2010). This suggests that Plexin-B2 may also function in this way. Interestingly Sema4A in this overexpression assay binds to all B family plexins and induces very similar percentages of growth cone collapse among cells expressing either Plexin-B1, B2, or B3 (Yukawa et al. 2010), suggesting that these genes can be redundant in their function.

1.7D. Plexin-B2 and c-Met

Plexin-B2, similar to Plexin-B1, binds to both c-Met and Ron, members of the scatter factor receptor family, although binding to Met is stronger (Conrotto et al. 2004). Studies of Plexin-B1 with c-Met have demonstrated that this interaction regulates invasive ability in NIH3T3 cells in a ligand dependant manner (Conrotto et al. 2004). The outcome of the interaction between Plexin-B2 with c-Met or Ron has not yet been studied.

1.7E. Plexin-B2 Binds PLCγ

Plexin-B2 has been shown to bind to PLCγ1 in the breast cancer cell line MCF-7 upon binding to its ligand Sema4C, but not in the breast cancer cell line MDA-MB-468, which lacks Erb-2 expression (Swiercz et al. 2009). Interestingly PLCγ1 is more expressed in the nervous system, and PLCγ2 in the immune, suggesting PLCγ expression may regulate nervous and immune cell differences in Rho activation downstream of Plexin-B2 (Swiercz et al. 2009). The downstream consequences of Plexin-B2 interacting with PLCγ are unproven, but likely result in the upregulation of the RhoA pathway, similar to Plexin-B1.

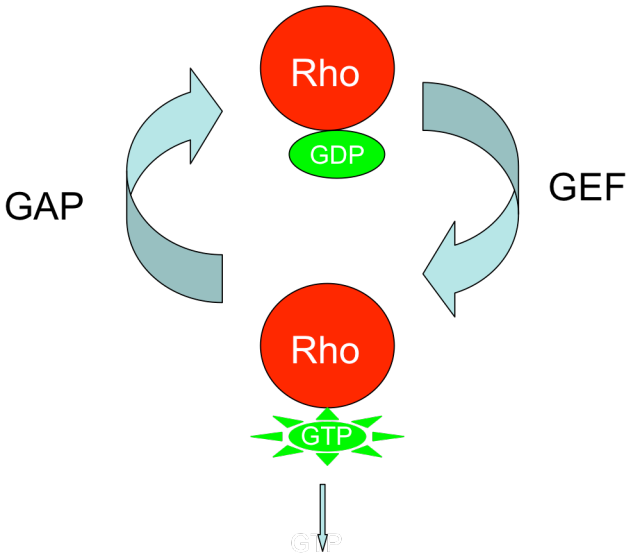
1.7F. Plexin-B2 in the Immune System

Plexin-B2 has been identified as a gene that is upregulated in T-dependent but not T-independent germinal centers. However the biological function of the upregulation of Plexin-B2 in germinal centers has not yet been elucidated. The research presented in this thesis explores the expression and function of Plexin-B2 in the immune system. Our studies show that in the innate immune system Plexin-B2 is dynamically expressed on macrophages

and dendritic cells and plays a role in cell motility, modulation of RhoGEFs, and cytokine response.

1.8 Conclusions

Plexins and semaphorins mediate many cell processes critical to the immune system including cell-cell contact, migration, and cytokine secretion. The B family of plexins includes Plexin-B1, B2, and B3. Plexin-B1 has been linked to regulation of the above processes through regulation of the actin cytoskeleton and its modulators, the Rho family of GTPases. The data presented here demonstrate the role of another B family member, Plexin-B2, in the immune system. Plexin-B2 is found on macrophages and dendritic cells and affects the Rho family of GTPases and cell motility.



Cytoskeletal changes, cell division, vesicle transport, cell cycle progression, modulation of gene expression

Figure 1.1

Figure 1.1: Rho family GTPases are controlled by GAPs and GEFs.

Rho family are intrinsic GTPases that modulate between GTP bound, active states and GDP bound inactive states to control downstream activities, the majority of which involve regulation of the actin cytoskeleton. The binding state of Rho proteins are modulated by GEFs, which exchange GDP for GTP and turn the protein "on", and GAPs, which accelerate the intrinsic GTPase capability of Rho family proteins and switch them to an inactive, GDP-bound "off" state.

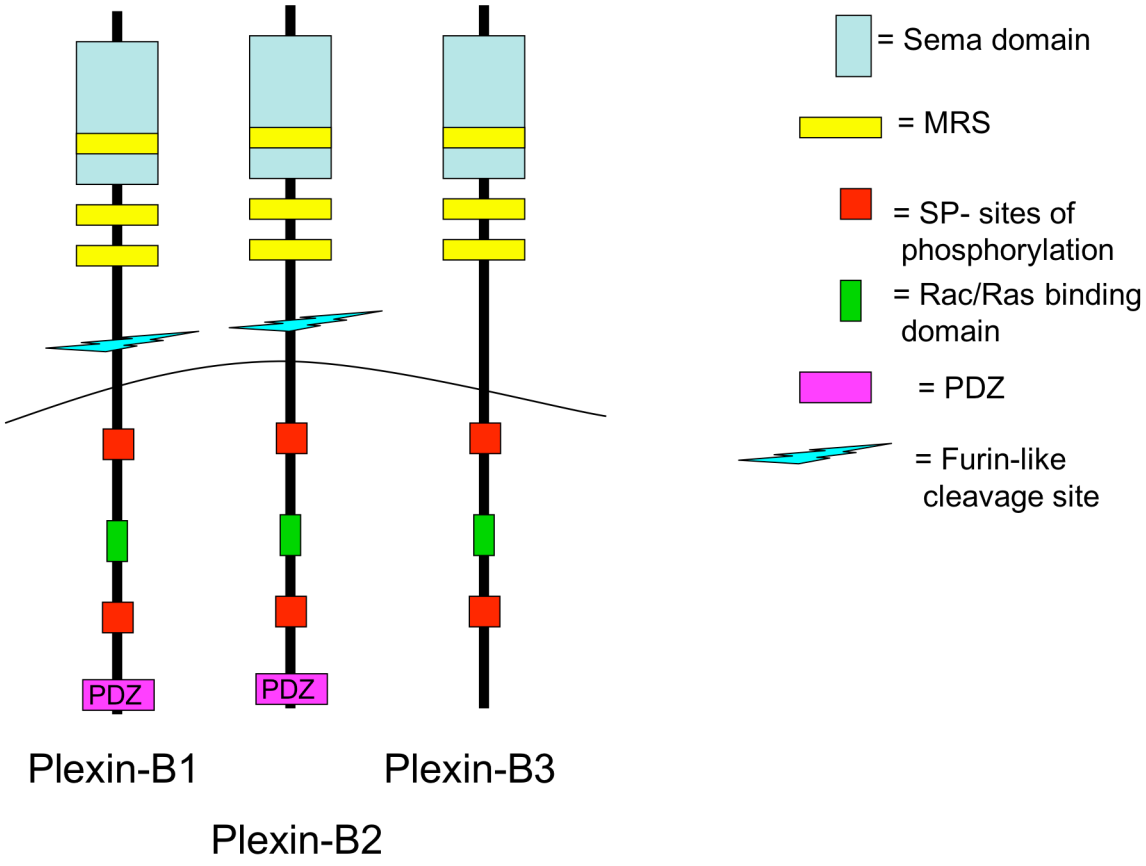

= Sema domain
= MRS
= SP- sites of phosphorylation
= Rac/Ras binding domain
= PDZ
= Furin-like cleavage site
PDZ
PDZ
Plexin-B1
Plexin-B3
Plexin-B2

Figure 1.2

Figure 1.2: Structure of the B family plexins.

Plexin-B1, Plexin-B2, and Plexin-B3 contain a conserved, 500 amino acid extracellular domain, and three extracellular MRS domains that are common to all plexins. Plexin-B1 and B2 are distinct from other plexins in containing furin-like cleavage sites, which may mediate extracellular cleavage and release of a soluble form of Plexin-B1 and Plexin-B2. Intracellularly, Plexin-B1-B3 contain a Rac/Ras binding domain that is flanked by two SP domains, which contain phosphorylation sites. Plexin-B1 and B2 contain an intracellular PDZ domain that can dimerize with PDZ domains of RhoGEFs and facilitate the upregulation of Rho. Figures were adapted from Tamagnone et al. (Tamagnone et al. 1999) and data from the National Center for Biotechnology Information (NCBI).

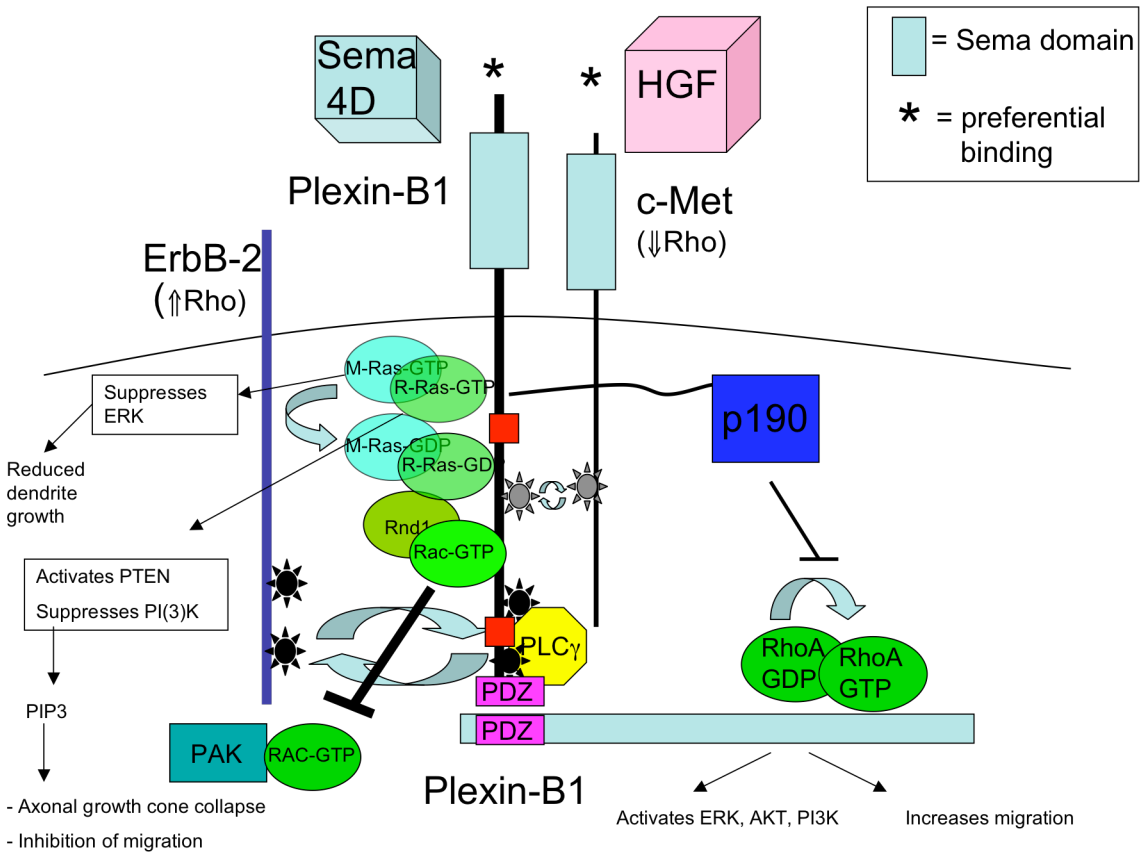

Sema 4D
*
*
HGF
= Sema domain
* = preferential binding
Plexin-B1
c-Met
(⇓Rho)
ErbB-2
(⇑Rho)
M-Ras-GTP
R-Ras-GTP
Suppresses ERK
M-Ras-GDP
R-Ras-GDP
p190
Reduced dendrite growth
Rnd1
Rac-GTP
Activates PTEN
Suppresses PI(3)K
PLCγ
RhoA GDP
RhoA GTP
PIP3
PDZ
PDZ
PAK
RAC-GTP
Plexin-B1
- Axonal growth cone collapse
- Inhibition of migration
Activates ERK, AKT, PI3K
Increases migration

Figure 1.3

Figure 1.3: Plexin-B1 regulates the Rho family of GTPases.

Ligand independent functions of Plexin-B1: Plexin-B1 binds to c-Met. c-Met can be phosphorylated by either binding to its ligand HGF or by Plexin-B1 binding to its ligand Sema4D (Giordano et al. 2002). Plexin-B1 has also been shown to interact with active, GTP bound Rac, leading to sequestration of Rac from its downstream effector PAK (Vikis et al. 2002).

Ligand dependent functions of Plexin-B1: Plexin-B1 acts as a GAP for R-Ras when Rnd1 is also in the Plexin-B1 complex (Oinuma et al. 2004). The Plexin-B1 mediated suppression of R-Ras induces inhibition of cell migration and suppression of PI(3)K pathway via activation of PTEN, resulting in inhibition of PIP_3 and axonal growth cone collapse (Oinuma et al. 2006; Oinuma et al. 2010). Plexin-B1 also serves as a GAP for M-Ras, which leads to downregulation of ERK activity and reduced migration and dendrite outgrowth in cortical neurons (Saito et al. 2009). This pathway operates when Plexin-B1 is bound to c-Met, and this binding partner is preferred over an alternative partner, ErbB-2. When Plexin-B1 is in complex with ErbB-2 the PI(3)K pathway is upregulated (Basile et al. 2005; Aurandt et al. 2006). Plexin-B1 phosphorylation by ErbB-2 mediates activation of RhoA and migration through creation of a PLCγ docking site on Plexin-B1 for RhoGEFs (Swiercz et al. 2008; Swiercz et al. 2009). Plexin-B1 activates Rho through binding of its PDZ domain to the PDZ domain of the RhoGEFs PDZ and LARG, leading to upregulated Rho, PI3K, AKT, ERK and migration (Driessens et al. 2002; Basile et al. 2005; Aurandt et al. 2006). The Plexin-B1 mediated activation of Rho can be downregulated by p190 (Barberis et al. 2005).

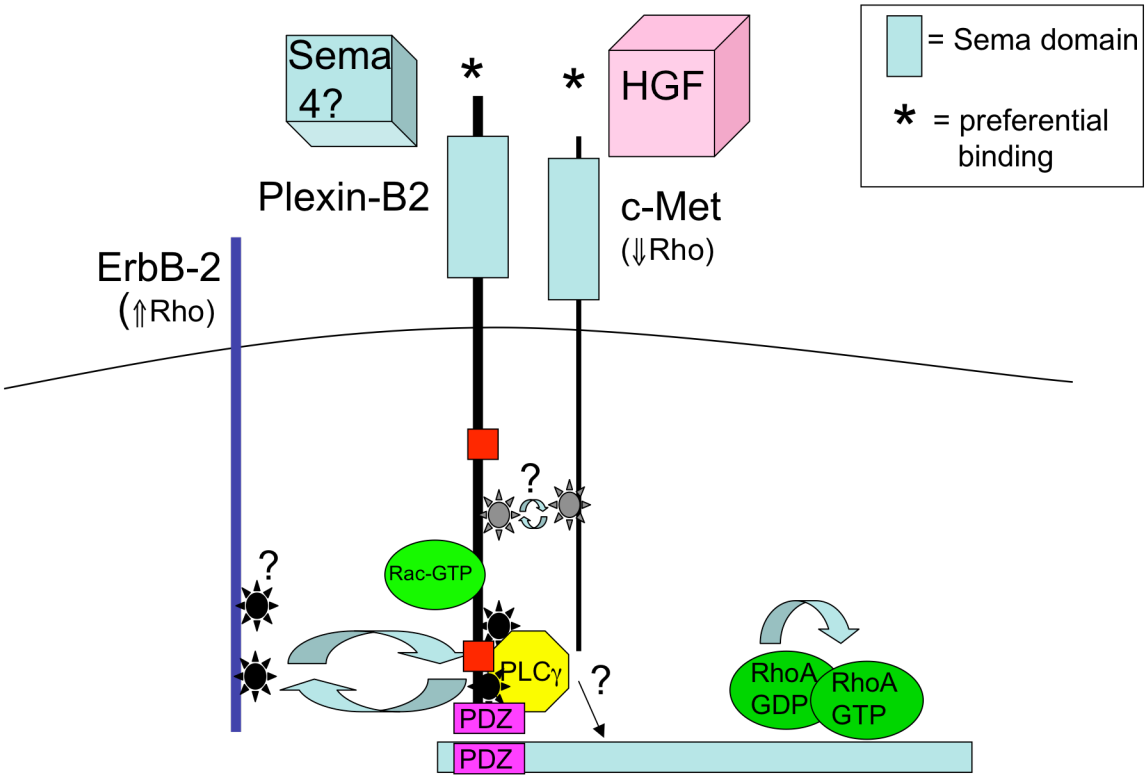

Sema
4?
*
*
HGF
= Sema domain
* = preferential binding
Plexin-B2
c-Met
(⇓Rho)
ErbB-2
(⇑Rho)
?
?
Rac-GTP
PLCγ
?
RhoA
GDP
RhoA
GTP
PDZ
PDZ

Figure 1.4

Figure 1.4: Plexin-B2 regulates the Rho family of GTPases.

Ligand independent functions of Plexin-B2: Plexin-B2 binds to c-Met (Conrotto et al. 2005). Plexin-B2 binds to active, GTP bound Rac (Driessens et al. 2001).

Ligand dependent functions of Plexin-B2: Plexin-B2, when synthetically dimerized by replacement of its extracellular domain with a known receptor, activates Rho through binding of its PDZ domain to the PDZ domain of the RhoGEFs PDZ and LARG (Perrot et al. 2002). PDZ and Rho facilitate the activation of Rho by facilitating the exchanging GTP for GDP. Plexin-B2 has been shown to bind to PLCγ upon binding to its ligand Sema4C, and possibly interacts with ErbB-2, as the phosphorylation of Plexin-B1 by ErbB-2 creates the docking site for PLCγ (Swiercz et al. 2009).

Chapter 2: Plexin-B2 Negatively Regulates Macrophage Motility and Rac and Cdc42 Activation

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ABSTRACT

Plexins are cell surface receptors widely studied in the nervous system, where they mediate migration and morphogenesis though the Rho family of small GTPases. More recently plexins have been implicated immune processes including cell-cell interaction, immune activation, migration, and cytokine production. Plexin-B2 facilitates ligand induced cell guidance and migration in the nervous system and induces cytoskeletal changes in overexpression assays through RhoGTPase, but its function in the immune system is unknown. This report shows that Plexin-B2 is highly expressed on cells of the innate immune system, including macrophages, conventional dendritic cells, and plasmacytoid dendritic cell. However it does not have detectable effects on the production of proinflammatory cytokines, phagocytosis of a variety of targets, or directional migration towards chemoattractants. Instead, *Plxnb2*$^{-/-}$ macrophages have greater cellular motility than wild type in the unstimulated state that is accompanied by more active, GTP-bound Rac and Cdc42. Studies have shown that B family plexins bind to only active Rac. The closely related B family member Plexin-B1 sequesters Rac from downstream signaling. These data presented here show that Plexin-B2 functions in macrophages as a negative regulator of Rac and Cdc42 and as a negative regulator of cell motility.

INTRODUCTION

The plexins are a family of nine transmembrane proteins that are grouped by homology into four subfamilies: A, B, C, and D (Tamagnone et al. 1999). All family members share an extracellular semaphorin domain and an intracellular plexin domain-containing tail that can mediate intracellular signaling. The plexins were originally identified in the nervous system (Ohta et al. 1992; Ohta et al. 1995), where they have been found to mediate diverse cell processes including axon guidance, neurogenesis, cell migration, cell proliferation and death. Plexins have also been found to function in other body systems including the reproductive, circulatory, endocrine, urinary, digestive, and immune system (reviewed in Kruger et al. 2005; O'Connor et al. 2008; Serini et al. 2009). Similar to other plexins, the B family of plexins were originally found in the nervous system (Tamagnone et al. 1999), and were later identified in the circulatory, endocrine, reproductive, urinary, digestive, respiratory, and immune systems (Regev et al. 2005; Harduf et al. 2007; Gomez Roman et al. 2008; Harduf et al. 2009; Li et al. 2009; Okuno et al. 2009; Pan et al. 2009; Stevens et al. 2010; Zielonka et al. 2010). The B plexin family is distinct from the A, C, and D plexins in the domains found in the intracellular tail. Two of the B family members, Plexin-B1 and Plexin-B2, contain an intracellular domain with a PDZ motif [post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)] (Aurandt et al. 2002; Driessens et al. 2002; Hirotani et al. 2002; Perrot et al. 2002; Swiercz et al. 2002; Basile et al. 2004).

In contrast to a paucity of studies on Plexin-B2, Plexin-B1 has been found in the immune system where it mediates processes similar to its function in the nervous system. Plexin-B1 is required for the optimal migration of monocytes and dendritic cells and

proliferation and survival of B cells (Granziero et al. 2003; Chabbert-de Ponnat et al. 2005). The mechanisms mediating these effects of Plexin-B1 in the immune system are unknown, but in other cell types the phenotypic effects of Plexin-B1 have been attributed to its role as a regulator of the Rho family of GTPases (Driessens et al. 2001; Aurandt et al. 2002; Driessens et al. 2002; Swiercz et al. 2002; Oinuma et al. 2004; Saito et al. 2009). The Rho family of GTPases functions to regulate actin dynamics (Etienne-Manneville et al. 2002; Mulloy et al. 2010). Plexin-B1 has been shown to regulate Rho upon stimulation by binding to PDZ-Rho and LARGE (Driessens et al. 2001; Aurandt et al. 2002; Driessens et al. 2002; Swiercz et al. 2002). Plexin-B1, as well as Drosophila Plexin-B, have been shown to bind directly to the active GTP-bound form of the GTPase Rac but not the inactive, GDP bound form (Vikis et al. 2000; Driessens et al. 2001; Hu et al. 2001). The downstream consequences of B family plexins binding to active Rac are not completely understood. In overexpression studies performed in HEK293 cells, Plexin-B1- Rac-GTP binding has been shown to sequester active Rac from its downstream effector p-21-activated kinase (PAK), which leads to increased cell surface expression of Plexin-B1 (Vikis et al. 2000). In Drosophila neurons, which have only one B family plexin, Plexin B binds to and down regulates Rac through an unknown mechanism (Hu et al. 2001).

Plexin-B2, the focus of our study, is much less studied than Plexin-B1. In overexpression studies Plexin-B2 has been found to regulate the GTPase Rho (Perrot et al. 2002). When Plexin-B2 is synthetically stimulated by replacement of its extracellular domain, the intracellular PDZ domain of Plexin-B2 binds to the PDZ domain of the RhoGEFs (guanine nucleotide exchange factors) PDZ-RhoGEF and LARGE (leukemia associated RhoGEF), leading to the activation of Rho and the formation of stress fibers in

fibroblast (Perrot et al. 2002). Studies of Plexin-B2 in the mouse nervous system have demonstrated that Plexin-B2 is required for normal development during embryogenesis. *Plxnb2*$^{-/-}$ embryo brains have defects in cortical patterning and in cell guidance of several cell types, resulting in neural tube closure defects and exencephaly (Friedel et al. 2007; Hirschberg et al. 2010). In the immune system, the function of Plexin-B2 has not been delineated, although Plexin-B2 has been identified on B cells as a marker of T cell dependent germinal center formation and on an undefined population of CD11b^{+} cells in the mouse liver (Yu et al. 2008; Zielonka et al. 2010). The function of Plexin-B2 on these cells types has not been reported.

This report shows that Plexin-B2 is most highly expressed by macrophages, conventional dendritic cells (cDC), and plasmacytoid dendritic cells (pDCs) and sought to determine the function of Plexin-B2 on these cells. To explore the effects Plexin-B2 on the immune system, we created fetal liver chimeric mice to reconstitute the immune system of wild type mice with *Plxnb2*$^{-/-}$ cells because the *Plxnb2*$^{-/-}$ mice do not survive post-partum. Reconstitution of the immune system with *Plxnb2*$^{-/-}$ cells has similar efficiency as reconstitution with wild type cells providing a feasible system for this study. The results show that *Plxnb2*$^{-/-}$ macrophages are not defective in their ability to secrete the inflammatory cytokines TNFα or IL-6, undergo phagocytosis of fluorescent beads, bacteria, or antibody bound T cells, or migrate towards chemokines CXCL12 and colony stimulating factor (CSF). However, *Plxnb2*$^{-/-}$ macrophages show a significant increase in cell mobility compared to wild type in steady state. Additionally *Plxnb2*$^{-/-}$ cells have higher levels of the active forms of the small Rho GTPases Cdc42 and Rac. These data show that Plexin-B2 is

transmembrane negative regulator of Rac and Cdc42 and modulates cell velocity in macrophages.

RESULTS

Plexin-B2 is expressed in the immune system.

We and others have previously shown that Plexin-A1 is found on dendritic cells in the mouse immune system and is required for optimal stimulation of T cells and for proper formation of T cell-dendritic cell conjugates by influencing actin polarization (Wong et al. 2003; Eun et al. 2006; O'Connor et al. 2008; Takamatsu et al. 2010). These results prompted us to ask if other plexins were expressed in the immune system. *In silico* analysis of the BioGPS database (Wu et al. 2009) demonstrates that human and mouse Plexin-B2 is highly expressed on macrophages, cDCs, and pDCs, with much less to little found on B and T cells (Fig. 2.1A, B). This expression pattern was analyzed in the mouse at the protein level by flow cytometry (Fig. 2.1C). Plexin-B2 is highly expressed on $B220^+$ PDCA-1^+ pDCs and $CD11c^+$ $B220^-$ cDCs in spleen and bone marrow. Plexin-B2 is also expressed moderately on splenic macrophages and higher on $F4/80^+$ macrophages. In addition bone marrow $F4/80^+$ cells show two populations based on Plexin-B2 expression. Bone marrow-derived $B220^+$ B cells also express a higher amount of Plexin-B2 than splenic B cells. The low levels of B cell Plexin-B2 transcript detected by *in silico* studies (Fig. 2.1A, B) and the much higher protein expression (Fig. 2.1C) might reflect post-transcriptional control of Plexin-B2 in B cells. Very little Plexin-B2 was detected on TCR^+CD4^+ T cells, TCR^+CD8^+ T cells, $NK1.1^+$ natural killer (NK) cells, or $NK1.1^+TCR^+$ NK T cells consistent with the *in silico* studies.

Reconstitution of the immune system with Plxnb2$^{-/-}$ cells is equivalent to wild type.

Mice lacking Plexin-B2 (*Plxnb2*$^{-/-}$) have been previously shown to have a severe phenotype, exhibiting defects in neural tube closure, cerebellar disorganization and misregulated granule cell proliferation (Deng et al. 2007; Friedel et al. 2007). To study the immune system, it was necessary to perform fetal liver transplant from *Plxnb2*$^{-/-}$ donor backcrossed onto a C57BL/6 background for at least ten generations into C57BL/6 CD45.1^{+} recipients. We and others have shown that plexins can affect cell proliferation and homeostasis (Deaglio et al. 2004; Kumanogoh et al. 2005; Hu et al. 2007; O'Connor et al. 2008; Takamatsu et al. 2010; Takamatsu et al. 2010). Therefore we studied the ability of *Plxnb2*$^{-/-}$ cells to reconstitute the mouse immune system. In the spleen and bone marrow, the reconstituted percentage of B220^{+} CD11c^{-} (cDC), CD11c^{low} PDCA-1^{+} (pDC) and F4/80^{+} (macrophage) cells, which express high levels of Plexin-B2, are equivalent in recipients of wild type *Plxnb2*$^{-/-}$ fetal livers (Fig. 2.2A). This also holds true for B220^{+} CD11c^{-} (B), TCR^{+} CD4^{+}, and TCR^{+} CD8^{+} T cell percentages in the spleen and bone marrow (Fig. 2.2A). Furthermore, the total percentage of reconstituting donor (CD45.2^{+}) cells compared to recipient (CD45.1^{+}) is comparable for recipients of either *Plxnb2*$^{-/-}$ or wild type cells (Fig. 2.2B). These data suggest that Plexin-B2 does not affect cell proliferation or reconstitution of the mouse immune system in the fetal liver engraftment system used for the rest of the study.

Plexin-B2 does not affect cytokine response in macrophages.

Plexins and their semaphorin receptors have been shown to modulate cytokine secretion. Plexin-A1 is required for upregulation of IFNα after Toll-like Receptor (TLR)

stimulation (Watarai et al. 2008). Blocking antibodies to Plexin-B1 have been shown to abrogate Sema4D-induced cytokine modulation (Chabbert-de Ponnat et al. 2005). To determine if Plexin-B2 also contributes cytokine response after TLR signaling, the inflammatory cytokines TNF and IL-6 in cell supernatants of macrophages stimulated by the TLR ligands Poly(I:C) (polyinosinic-polycytidylic acid, TLR3), LPS (lipopolysaccharide, TLR4), and R837 (imidazoquinoline compound imiquimod, TLR7) were assessed (Fig.2.3A, B). In response to TLR ligands, *Plxnb2*$^{-/-}$ and wild type macrophages secreted comparable levels of TNFα and IL-6, indicating that Plexin-B2 does not play a role in the production or release of cytokines measured in this study.

Plexin-B2 negatively regulates cell motility.

Plexin-B2 has been shown in the nervous system to mediate cell guidance, migration, and proliferation These data prompted us to examine if Plexin-B2 could contribute to cell movement in macrophages. We compared the motility of *Plxnb2*$^{-/-}$ and wild type macrophages on glass bottom dishes using time-lapse microscopy. Three independent movies of *Plxnb2*$^{-/-}$ and wild type cells show that the mean velocity of *Plxnb2*$^{-/-}$ macrophages is significantly higher than wild type (Fig. 2.4A, B). We also stimulated macrophages with macrophage colony stimulating factor (M-CSF) and found that while wild type macrophage motility increased with stimulation, *Plxnb2*$^{-/-}$ macrophage motility remained constant. This indicates that Plexin-B2 serve as a motility brake in unstimulated cells, and does not affect motility in response to M-CSF stimulation.

We also explored the directional migration capacity of *Plxnb2*$^{-/-}$ macrophages in transwell assays. Macrophages have been demonstrated to migrate towards the attractive

chemokines M-CSF and stromal cell-derived factor-1 (CXCL12) (Kheir et al. 2005; Campana et al. 2009). The data show that migration of *Plxnb2*$^{-/-}$ macrophages to M-CSF and CXCL12is normal compared to wild type. This suggesting that while Plexin-B2 negatively regulates cell motility in steady state to prevent motility when cells are unstimulated, it does not regulate directed migration towards M-CSF and CXCL12 in macrophages.

Plexin-B2 Negatively Regulates Rac and Cdc42.

Previous studies with overexpressed Plexin-B2 in Swiss 3T3 cells have shown that Plexin-B2 binds to the active, GTP-bound form of Rac and contains a Rac binding motif in its intracellular tail (Driessens et al. 2001). Following the findings that Plexin-B2 affects cell velocity, we investigated if this regulation of motility could be related to Plexin-B2 dependent regulation of Rac. Rac and Cdc42 have been demonstrated to modulate cell migration and motility (Etienne-Manneville et al. 2002; Pankov et al. 2005; Heasman et al. 2008). The GTP binding state of Rac and Cdc42 was assessed using GST–PBD (glutathione S-transferase PAK1 p21-binding domain) beads to pull down activated Rac and Cdc42 from cell lysate followed by specific detection of each of the molecule by western blot (Sander et al. 1998; Arthur et al. 2001). In the unstimulated steady state, *Plxnb2*$^{-/-}$ macrophages repeatedly had more GTP bound Rac and Cdc42 than wild type (Fig. 2.5A). Following stimulation with M-CSF for ten minutes, wild type and *Plxnb2*$^{-/-}$ macrophages showed similar levels of active Rac and less Cdc42. This suggests that Plexin-B2 is a negative regulator of Rac and Cdc42 in the unstimulated state but not in the stimulated state. Studies with the closely related protein Plexin-B1 have shown that Plexin-B1 binding to Rac-GTP occurs in steady state (Driessens et al. 2001). Our data shows that Plexin-B2 is similar to

Plexin-B1 in that its effect on Rac but additionally found that the former also regulates Cdc42. We also examined the effect Plexin-B2 on the ERK (extracellular-signal-related-kinase) pathway, as previous studies of Plexin-B1 have shown that ERK is phosphorylated downstream of Plexin-B1 (Fig. 2.5B) (Aurandt et al. 2006). Phosphorylated ERK levels in *Plxnb2*$^{-/-}$ and wild type macrophages were equivalent in the steady state and in response to treatment with M-CSF. These data represent the first evidence that the endogenous effect of Plexin-B2 in unstimulated cells is to serve as a brake for Rac and Cdc42 activation.

Plexin-B2 does not affect macrophage phagocytosis.

The Rho family of GTPases regulate the process of phagocytosis, in which actin dynamics facilitate the cellular processes of membrane extension, phagocytic cup formation and closure, and particle uptake (reviewed in Fenteany et al. 2004). Rac and Cdc42 have been shown to mediate FcγR mediated phagocytosis in macrophages (Cox et al. 1997; Caron et al. 1998; Park et al. 2009). Plexin-C1, in response to viral semaphorin, has been shown to downregulate phagocytosis (Ji et al. 2009).

To test whether the negative regulation of Rac and Cdc42 by Plexin-B2 in macrophages has an effect on phagocytosis we performed experiments to examine the uptake of GFP-E. coli, latex beads, and antibody coated thymocytes (Fig. 2.6A, B, C). *Plxnb2*$^{-/-}$ or wild type macrophages were given either GFP-E. coli, fluorescent latex beads, or antibody coated thymocytes and assayed for cellular uptake using flow cytometry at different time points. Both *Plxnb2*$^{-/-}$ and wild type macrophages were capable of phagocytosing all treatment groups. However, the ability to phagocytose antibody coated thymocytes

repeatedly trended towards greater uptake of these thymocytes by *Plxnb2*$^{-/-}$ macrophages although this difference is not significant.

DISCUSSION

This is the first report to explore the expression of Plexin-B2 on macrophages, cDCs, and pDCs. We found that Plexin-B2 is not expressed on resting B cells in the spleen, in agreement with previously reported expression of Plexin-B2 in B cells within T-dependent germinal centers (Yu et al. 2008). By contrast, Plexin-B2 expression on T cells is consistent and it is not highly expressed in $CD4^+$ or $CD8^+$ splenic T cells or NK or NK T cells at the transcript and protein level. In the BioGPS cDNA database (Wu et al. 2009), Plexin-B2 is most highly expressed in the macrophage, cDC and pDC in the immune system. Our protein data confirms the database results. Interestingly, the same database shows Plexin-B1 has no immune system cDNA expression above median, and Plexin-B3 is most highly expressed in mast cells (data not shown). Other studies have demonstrated that Plexin-B1 is expressed in B cells, monocytes, and dendritic cells in humans (Granziero et al. 2003; Chabbert-de Ponnat et al. 2005). This suggests that expression level of different B family plexins is cell type specific and may be a way of regulating the function of B family plexins.

The functional analyses indicate that *Plxnb2*$^{-/-}$ macrophages have higher steady state velocity than wild type macrophages by live cell microscopy, suggesting that Plexin-B2 is a negative regulator of cell velocity. The data demonstrates that directional migration of both *Plxnb2*$^{-/-}$ and wild type macrophages in response to cytokines M-CSF or CXCL12 are equivalent, suggesting that stimulated, directional migration is not affected by Plexin-B2 with these chemokines. The majority of the studies of Plexin B family members have relied

on activation of the plexin receptor by its ligand or by synthetic dimerization. Here we show that endogenous, unstimulated Plexin-B2 regulates macrophage velocity in steady state. It is possible that Plexin-B2 may be dimerized in an autocrine fashion in our system by one of its reported ligands Sema4A, Sema4C, or Sema4D in steady state (Masuda et al. 2004; Deng et al. 2007; Yukawa et al. 2010; Zielonka et al. 2010). Under the scenario, the semaphorin ligand could be secreted by macrophages, or could be provided by neighboring cells in the culture. However, we are able to use a monoclonal antibody to stain for surface expression of Plexin-B2 indicating that either the anti-Plexin-B2 antibody binds to a portion of Plexin-B2 that is not occupied by ligand binding or that Plexin B2 is not bound to its ligand in our experimental conditions.

To explore the mechanism of Plexin-B2 negative regulation of cell velocity, GTP pull down assays were carried out to determine the amount of active Rac and Cdc42. The data show that in the unstimulated steady state, *Plxnb2*$^{-/-}$ cells have higher levels of the GTP bound, active forms of Cdc42 and Rac. Previous reports have shown that Plexin-B2 in its undimerized form binds to Rac-GTP in yeast and in bacterial overexpression studies (Driessens et al. 2001). Our data suggests that similar to Plexin-B1, Plexin-B2 may bind to RacGTP and function to sequester Rac from it downstream effectors (Vikis et al. 2002). In the absence of Plexin-B2, there is more active Rac, suggesting that Plexin-B2 might increase RacGAP (Rac GTPase accelerating protein) activity by serving as a RacGAP itself or recruits a RacGAP to the Plexin-B2 complex. Structural and biochemical studies of Plexin-B1 have shown that Plexin-B1 does not function as a RacGAP, suggesting that the Plexin-B1 modulation of Rac is through other mechanisms (Bouguet-Bonnet et al. 2008; Hota et al. 2009). The intracellular tails of Plexin-B1 and Plexin-B2 are similar (61% identical in their

amino acid sequence – data not shown) it is likely that Plexin-B2 also itself does not function as a RacGAP but instead mediates activity towards Rac through the modulation of other regulators in this pathway. Interestingly Plexin-B1 has been shown in yeast to not interact with Cdc42, while our data indicate that Plexin-B1 does modulate Cdc42 activation. This reveals a difference between Plexin-B1 and Plexin-B2, however species differences have to be taken into account for these differences. We observe the effects of Plexin-B2 on Rac and Cdc42 only in an unbound state.

This study revealed several functions that are not regulated by Plexin-B1. The reconstitution ratios of wild type or *Plxnb2*$^{-/-}$ fetal liver cells are similar in wild type recipients, and the percent of specific immune cell populations in the spleen and bone marrow reconstituted equivalently. This includes cell types that express the highest levels of Plexin-B2: the cDC, pDC, and macrophage. Numbers of macrophage cells derived from bone marrow cultures of *Plxnb2*$^{-/-}$ and wild type cells are equal, suggesting that Plexin-B2 does not contribute to detectable differences in immune system ontogeny or proliferation in this study.

Antibody-mediated phagocytosis is a Cdc42 dependent mechanism. Therefore we explored the phagocytic capacity of *Plxnb2*$^{-/-}$ macrophages compared to wild type. However, the findings show that *Plxnb2*$^{-/-}$ macrophages are capable of phagocytosis of GFP-E. coli, latex beads, and antibody coated thymocytes. Phagocytosis of GFP-E. coli and latex beads is similar for *Plxnb2*$^{-/-}$ and wild type macrophages. However, there was a trend of increased phagocytosis of antibody-coated thymocytes by *Plxnb2*$^{-/-}$ macrophages. Antibody-mediated phagocytosis has been shown to be dependant on Rac and Cdc42 (Caron et al. 1998), while bacteria bypass the need for activation of the small GTPases for cellular uptake (Gruenheid et

al. 2001). It is possible that the over activation of Rac and Cdc42 in the absence of Plexin-B2 leads to a modest upregulation of antibody-mediated phagocytosis, but not phagocytosis of E. coli and latex beads.

Previous reports have shown that Plexin-B1 and other plexins can influence cytokine production in monocytic cells (Holmes et al. 2002; Chabbert-de Ponnat et al. 2005). However this study shows that TNFα and IL-6 production and secretion in response to TLR ligands is similar between wild type and *Plxnb2*$^{-/-}$ macrophages. Thus in our system cytokine secretion is not affected by the absence of Plexin-B2 signaling. It is possible that dimerization of Plexin-B2 or stimulation by its ligand could show that Plexin-B2 has a role in macrophage cytokine secretion and should be addressed in future studies.

In summary, this study shows that Plexin-B2 is highly expressed in the immune system on cells of monocytic-myeloid lineage, including the cDC, pDC, and macrophage. Plexin-B2 has been shown previously to bind to only to the activated form of Rac, but the physiological consequence of Plexin-B2 Rac-GTP binding is unknown. We show that *ex-vivo Plxnb2*$^{-/-}$ macrophages have higher levels of activated Rac and Cdc42 and increased cell velocity in steady state. Our data suggest that the function of Plexin-B2 in steady state is to negatively regulate Rac and Cdc42 and to maintain a brake on cell motility in unstimulated cells.

Acknowledgements

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MATERIALS AND METHODS

Mice

C57BL/6 (CD45.2) and congenic C57BL/6 (CD45.1) mice were purchased from National Cancer Institute (Boston, MA). *Plxnb2*$^{-/-}$ mice were provided by Dr. Marc Tessier-Lavigne (Friedel et al. 2007) (Stanford) and were backcrossed with C57BL/6 mice at least 10 generations at the University of North Carolina Chapel Hill. Mice were used at six to eight weeks of age and were housed in a pathogen-free barrier facility at the University of North Carolina Chapel Hill. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and were performed in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. *Plxnb2*$^{+/-}$ mice were crossed to obtain *Plxnb2*$^{-/-}$ and wild type fetal livers from stage E14 pups. Fetal liver cells were then injected intravenously into lethally irradiated C57BL/6 CD45.1 to reconstitute the immune system and analyzed 6-10 weeks post reconstitution (Godin et al. 2002).

Cell culture

Macrophages were generated by bone marrow culture in L929 media. In brief, mouse femurs and tibias were removed from 6-8 week old mice, cleaned, and aspirated to remove bone marrow. Cells were cultured in L929 for six days. Macrophages were harvested and replated in complete DMEM media [DMEM, 10% heat inactivated fetal bovine serum, non-essential amino acids, L-glutamine, sodium pyruvate, and penicillin/streptomycin (P/S)], and rested overnight before experiments.

Reagents

Poly(I:C), Ultrapure LPS, and Imiquimod (R837) were from InvivoGen. M-CSF is from R&D Systems (Minneapolis, MN). Antibodies for western blotting included Rac1 (C-14; sc-217), Cdc42 (B-8; sc-8401), phospho-ERK1/2 (Thr202/Thr204) (197G2; 4377) from Santa Cruz Biotechnology (Santa Cruz, CA) and GAPDH (MAB374) from Millipore (Billerica, MA). Secondary goat α mouse-HRP (horse radish peroxidase) and goat α rabbit-HRP antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA). Beads for assays of GTP-bound Rac1 and Cdc42 glutathione-sepharose (GST) bead conjugated with Pak1 binding domain (Rac1 and Cdc42) were kindly provided by Dr. Keith Burridge (University of North Carolina, Chapel Hill).

Flow cytometry

Antibodies used for flow cytometry included: B220 (RA3-6B2), CD45.2 (104), CD45.1 (A20), CD4 (L3T4), CD8 (Ly-2), TCR (H57-597), CD11b (M1/70), CD11c (N418), NK1.1 (NKR-P1C) PDCA-1 (BST2, CD317) and Plexin-B2 (3E7) from eBioscience (San Diego, CA). Single cell suspensions of spleen and bone marrow were lysed in ACT to remove red blood cells, washed and resuspended in FACS buffer (1X PBS and 2% FBS) at $1x10^{6}$ cells per well and stained with antibody combinations. All experiments were performed on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) or Cyan (Dako, Carpinteria, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Live Cell Microscopy

$1x10^4$ cells were plated on glass bottom dishes (MatTek Ashland, MA P35G-1.5-10-C). Cells were imaged on a Nikon Biostation (Belmont, CA) with a 20X objective. Cell velocity was analyzed using manual tracking in ImageJ (Abramoff 2004). Images were collected every 5 minutes for a total time of 2.5 hours for unstimulated cells or following treatment with 50ng/ml M-CSF. For each treatment three separate movies were filmed and 45 cells scored for velocity. n=3 mice per group.

Detection of GTP-bound Rac1, Cdc42, and pERK

Cells were plated at $2x10^6$ cells per well in six well plates and treated with 50ng/ml M-CSF or media for the indicated times. Assays for GTP-bound Rac1 and Cdc42 were performed as described (Noren et al. 2003). Cells were lysed and precipitated using GST-PBD beads for Rac and Cdc42. Bead bound proteins and cell lysate were resolved on a NuPAGE (Invitrogen Carlsbad, CA) gel and were transferred onto nitrocellulose membranes. Membranes were blocked with 10% milk and probed with primary antibodies against Rac1 and Cdc42 followed by appropriate secondary HRP conjugated antibody for detection. For detection of pERK cells were plated as above, treated with 50ng/ml M-CSF for the indicated times, lysed and resolved on a NuPage (Invitrogen Carlsbad, CA) gel.

Phagocytosis

Macrophages were plated at $1x10^5$ cells per well in a 96 well non-tissue culture treated plate in 100ul of macrophage media without antibiotics. Cells were rested overnight and then treated with $1x10^7$ GFP E. coli (green fluorescent protein expressing *Eshericia coli* kindly provided by Dr. Glenn Matoshima, University of North Carolina Chapel Hill), $1x10^7$ fluorescent latex beads (Invitrogen F13080, Carlsbad, CA), or $1x10^6$ CFSE

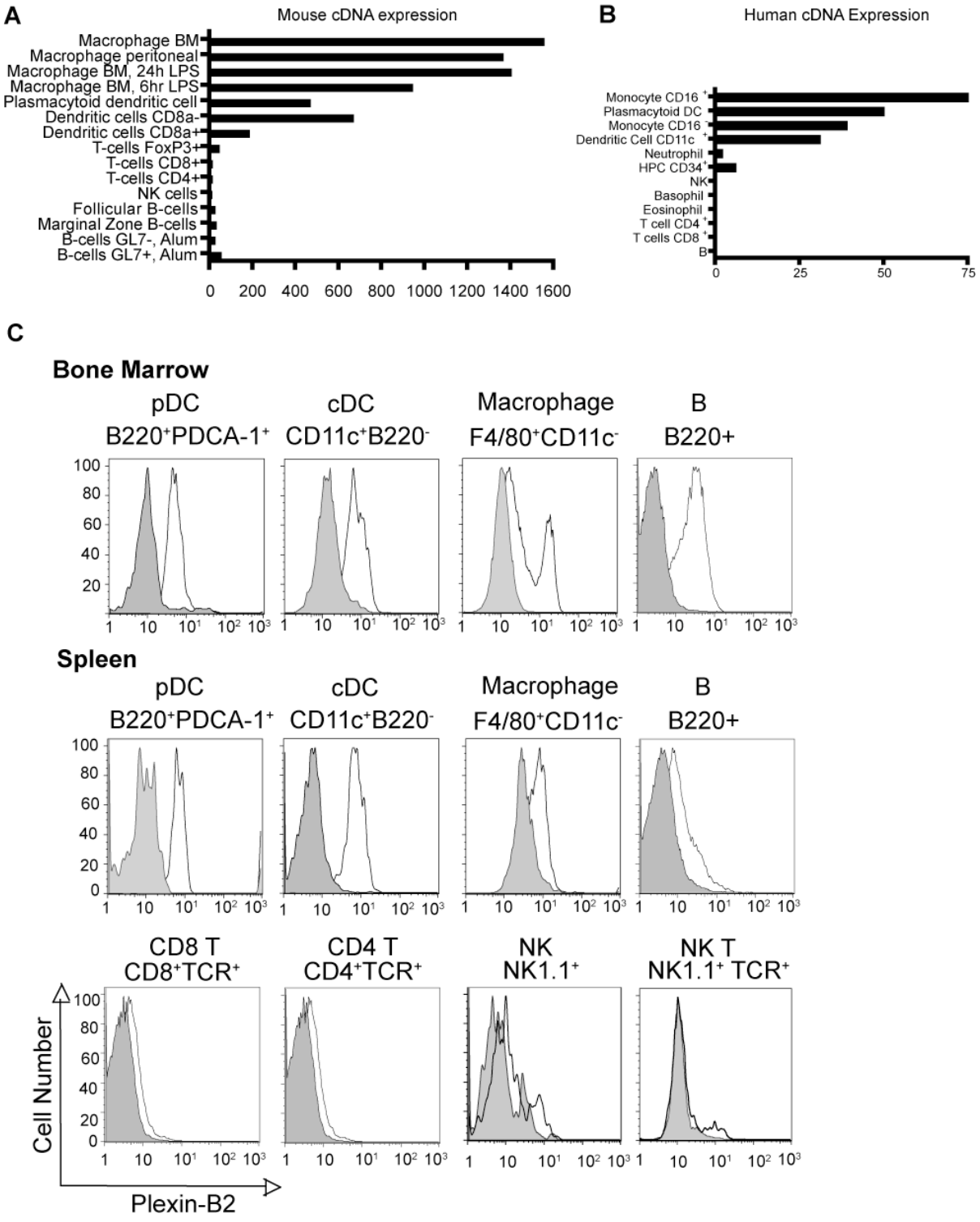
(carboxyfluorescein diacetate succinimidyl ester, Invitrogen, Carlsbad, CA) labeled, antibody labeled (CD45.2, mouse IgG2a) thymocytes as previously described (Scott et al. 2001). Cells were washed with media, extracellular fluorescence quenched with 0.2% Trypan Blue (Sigma T8154, St. Louis, MO), fixed in 0.1% EM-grade formaldehyde and analyzed by flow cytometry for percent of fluorescent positive cells.

ELISA

Macrophages were plated at $2x10^5$ cells per well in a 96 well plate and stimulated overnight. Cell supernatants were quantified using the ELISA kits for mouse TNF-α (555268) or IL-6 (555240) (BD Biosciences San Jose, CA).

Statistical analysis

Statistical significance was determined with two-tailed Student's t test. All *p* values less than 0.05 were considered significant.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Macrophage peritoneal
Macrophage BM, 24h LPS
Macrophage BM, 6hr LPS
Plasmacytoid dendritic cell
Dendritic cells CD8a-
Dendritic cells CD8a+
T-cells FoxP3+
T-cells CD8+
T-cells CD4+
NK cells
Follicular B-cells
Marginal Zone B-cells
B-cells GL7-, Alum
B-cells GL7+, Alum
0 200 400 600 800 1000 1200 1400 1600
B
Human cDNA Expression
Monocyte CD16+
Plasmacytoid DC
Monocyte CD16-
Dendritic Cell CD11c+
Neutrophil
HPC CD34+
NK
Basophil
Eosinophil
T cell CD4+
T cells CD8+
B
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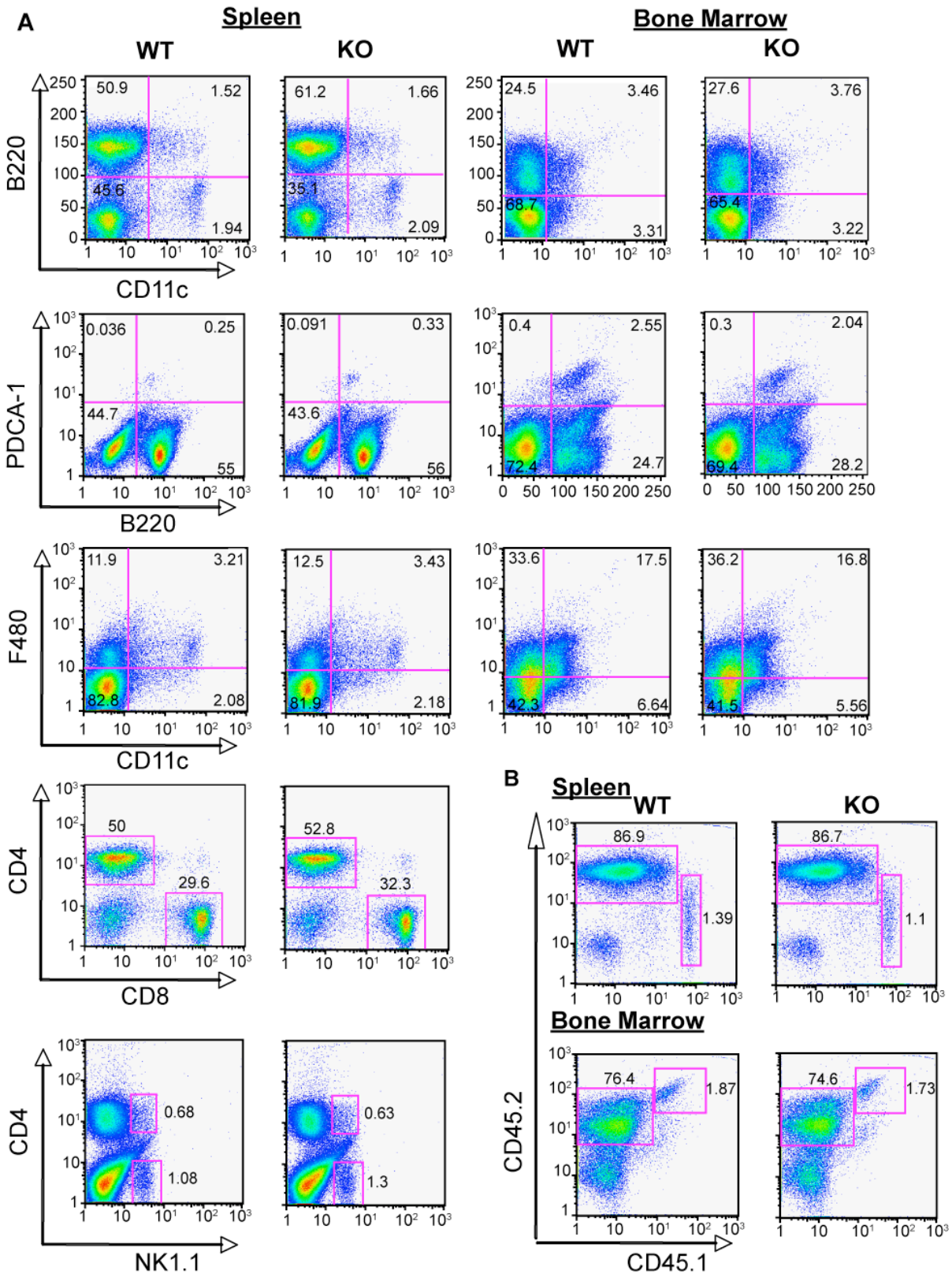
C
Bone Marrow
pDC B220+PDCA-1+
cDC CD11c+B220-
Macrophage F4/80+CD11c-
B B220+
Spleen
pDC B220+PDCA-1+
cDC CD11c+B220-
Macrophage F4/80+CD11c-
B B220+
CD8 T CD8+TCR+
CD4 T CD4+TCR+
NK NK1.1+
NK T NK1.1+ TCR+
Cell Number
Plexin-B2

Figure 2.1

Figure 2.1. Plexin-B2 is expressed in the human and mouse immune system.

A) cDNA expression level of *Plxnb2* in mouse immune cells from BioGPS database (Wu et al. 2009). B) cDNA expression level of *Plxnb2* in human immune cells. C) Plexin-B2 protein expression by flow cytometry of *ex-vivo* mouse spleen and bone marrow cells. pDCs were gated on $B220^+$ PDCA-1^+ cells. cDCs were gated on $CD11c^+$ $B220^-$ cells. Macrophages were gated on $F4/80^+$ $CD11c^-$ cells. B cells were gated on $B220^+$ cells. T cells were gated on TCR^+ cells. NK cells were gated on $NK1.1^+$ TCR^- cells, and NK T cells on $NK1.1^+TCR^+$ cells. Wild type fetal liver reconstituted mice are indicated by unfilled histogram and $Plxnb2^{-/-}$ reconstituted mouse by filled histogram. Results are representative of at least four experiments, n=8 per group.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Spleen
Bone Marrow
WT
KO
WT
KO
B220
CD11c
50.9
1.52
45.6
1.94
61.2
1.66
35.1
2.09
24.5
3.46
68.7
3.31
27.6
3.76
65.4
3.22
PDCA-1
B220
0.036
0.25
44.7
55
0.091
0.33
43.6
56
0.4
2.55
72.4
24.7
0.3
2.04
69.4
28.2
F480
CD11c
11.9
3.21
82.8
2.08
12.5
3.43
81.9
2.18
33.6
17.5
42.3
6.64
36.2
16.8
41.5
5.56
CD4
CD8
50
29.6
52.8
32.3
CD4
NK1.1
0.68
1.08
0.63
1.3
B
Spleen
WT
KO
86.9
1.39
86.7
1.1
Bone Marrow
CD45.2
CD45.1
76.4
1.87
74.6
1.73

Figure 2.2

Figure 2.2. Immune system reconstitution with *Plxnb2*$^{-/-}$ fetal livers is similar to wild type.

Flow cytometry of reconstitution of congenic CD45.1^{+} mice with wild type and *Plxnb2*$^{-/-}$ fetal livers (CD45.2^{+}) in the splenic and bone marrow compartments six weeks post transplant. A) cDCs are designated as B220-/CD11c+ cells, pDCs as CD11c^{+} B220^{+}, and B cells as B220^{+} CD11c^{-}. pDCs were further defined as CD11c^{low} and PDCA-1^{+}. Macrophages were defined as F4/80^{+} cells. T cells were first gated on TCR^{+} cells, and then divided into subpopulations by the CD4^{+} and CD8^{+} markers. NK cells were defined as NK1.1 +/CD4- and NKT cells as NK1.1+CD4+. B) Total donor reconstitution in the spleen and bone marrow were similar between wild type and *Plxnb2*$^{-/-}$ mice. CD45.2 marked the donor, Ly5.1^{+} cells. CD45.1 marked the residual host, Ly5.2^{+} cells. Figures are representative plots of four separate experiments. n=5 mice per group.

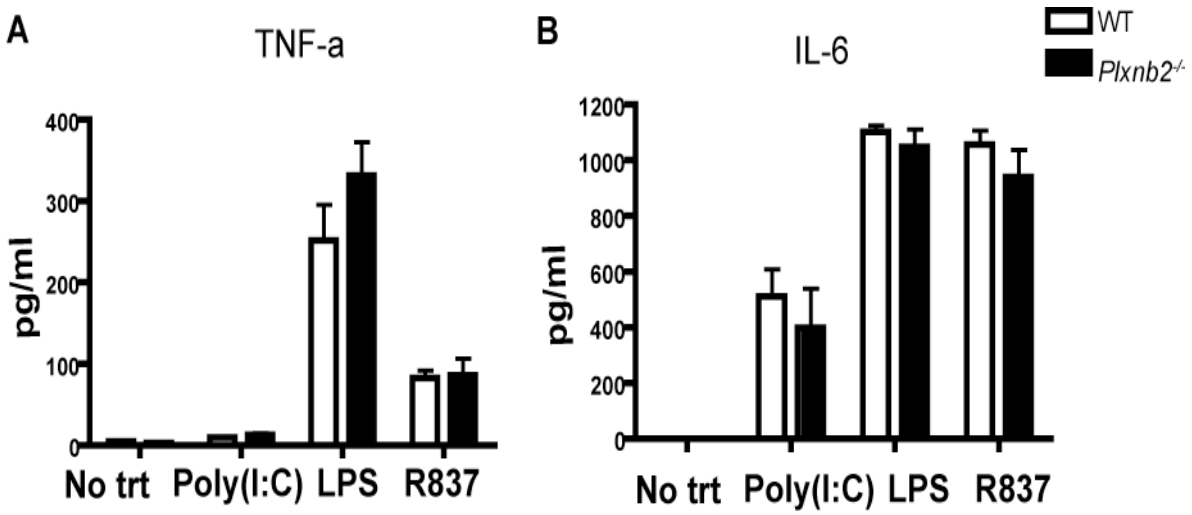
A
TNF-a
pg/ml
400
300
200
100
0
No trt
Poly(I:C)
LPS
R837
B
IL-6
WT
Plxnb2-/-
pg/ml
1200
1000
800
600
400
200
0
No trt
Poly(I:C)
LPS
R837

Figure 2.3

Figure 2.3: Plexin-B2 does not affect cytokine secretion.

Wild type and *Plxnb2*$^{-/-}$ macrophages were incubated for 16 hours with TLR ligands Poly(I:C) (10 ug/ml), LPS (1ug/ml) , and R837 (4 ug/ml). Supernatants were collected at the 16 hour time point and assessed for secretion of A) TNF and B) IL-6 by ELISA. Graphs are representative of three independent experiments, n=3 mice per group.

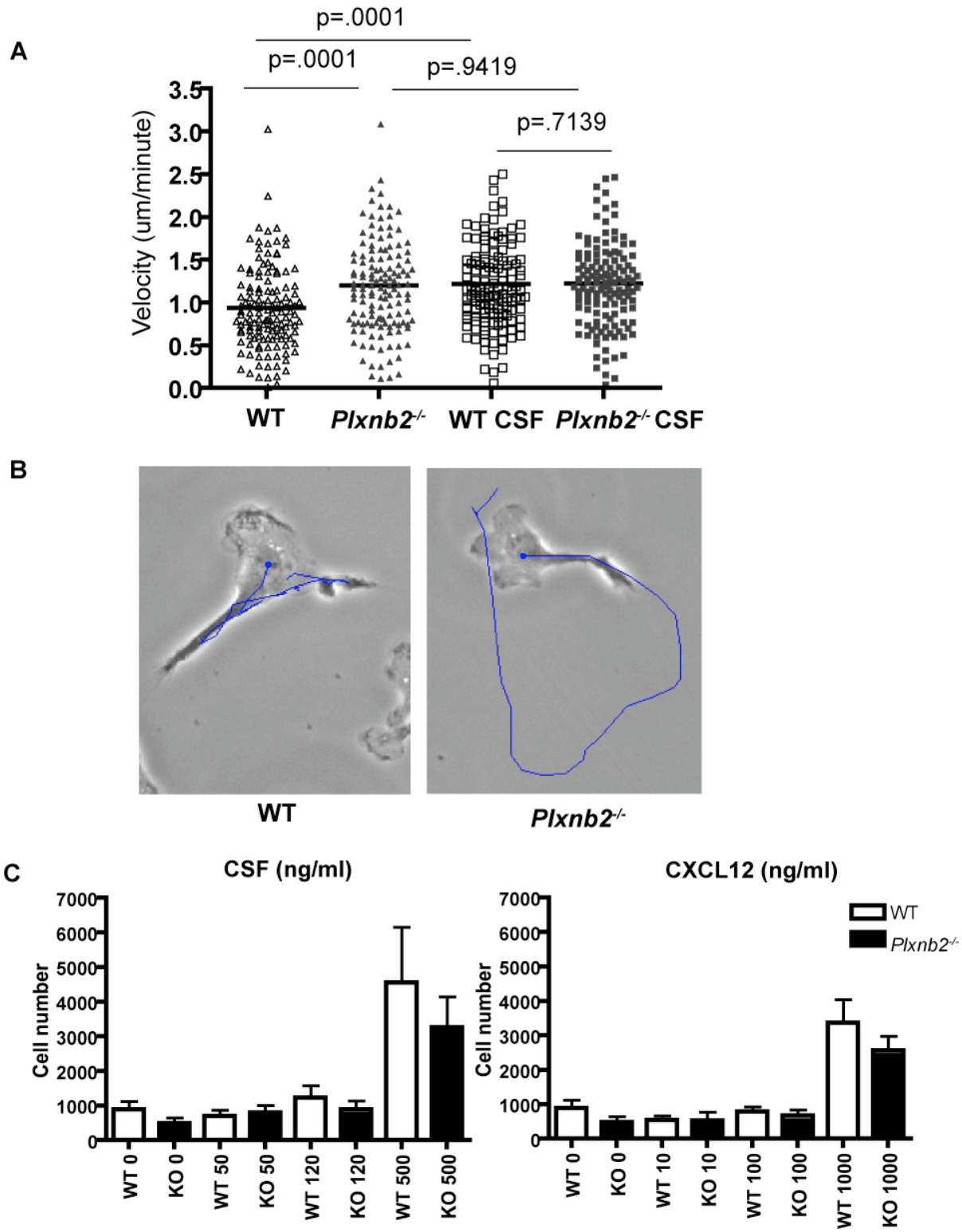
A
p=.0001
p=.0001
p=.9419
p=.7139
Velocity (um/minute)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
Plxnb2-/-
WT CSF
Plxnb2-/- CSF
B
WT
Plxnb2-/-
C
CSF (ng/ml)
CXCL12 (ng/ml)
Cell number
7000
6000
5000
4000
3000
2000
1000
0
WT 0
KO 0
WT 50
KO 50
WT 120
KO 120
WT 500
KO 500
WT 10
KO 10
WT 100
KO 100
WT 1000
KO 1000
WT
Plxnb2-/-

Figure 2.4

Figure 2.4. *Plxnb2*$^{-/-}$ cells have higher velocity than wild type macrophages.

A) Representative cell movement tracks over a 2.5 hour time period on untreated wild type or *Plxnb2*$^{-/-}$ macrophages. B) Combined velocity scores of wild type and *Plxnb2*$^{-/-}$ bone marrow macrophages in untreated or treated (50ng/ml M-CSF) groups. Cell velocity was scored in 45 cells per group per experiment. The experiment was repeated three separate times using cells from different mice in each experiment. C) Macrophage transwell migration towards CSF and CXCL12. Cells were placed in the upper chamber and chemokines in the lower chamber of migration plates and allowed to migrate for four hours. Cells were quantified and normalized to a standard curve of each genotype. Graphs are representative of at least three independent experiments. n= 4 mice per group.

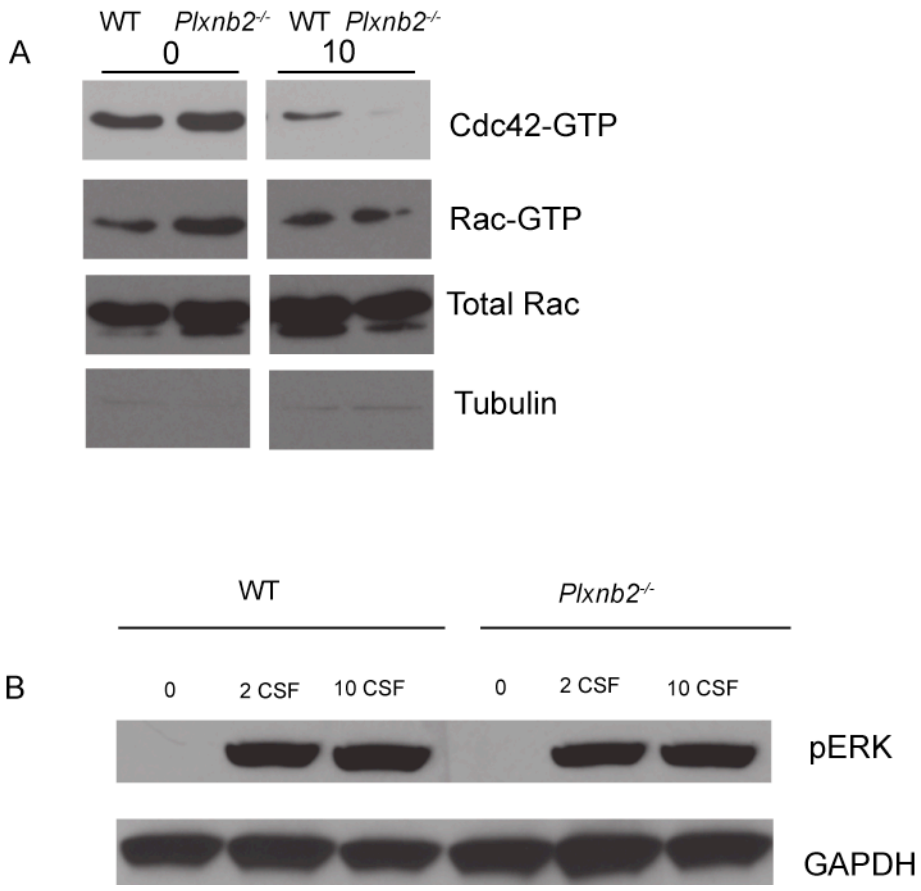
A
WT
Plxnb2-/-
WT
Plxnb2-/-
0
10
Cdc42-GTP
Rac-GTP
Total Rac
Tubulin
B
WT
Plxnb2-/-
0
2 CSF
10 CSF
0
2 CSF
10 CSF
pERK
GAPDH

Figure 2.5

Figure 2.5: Plexin-B2 inhibits active Rac and Cdc42.

A) WT and *Plxnb2*$^{-/-}$ macrophages were plated and rested overnight and assessed for RacGTP, Cdc42GTP, total Rac and total Cdc42 using a GST-PBD pulldown assay followed by western blot. B) Western blot of ERK activation as assessed by phospho-ERK level in untreated cells, as well as in response to 2 and 5 minutes of treatment with CSF (50 ng/ml) and 20 minutes of treatment with LPS (1 ug/ml). Total protein level was determined by western blot of GAPDH. Results are representative of four separate experiments. n= 4 mice per group.

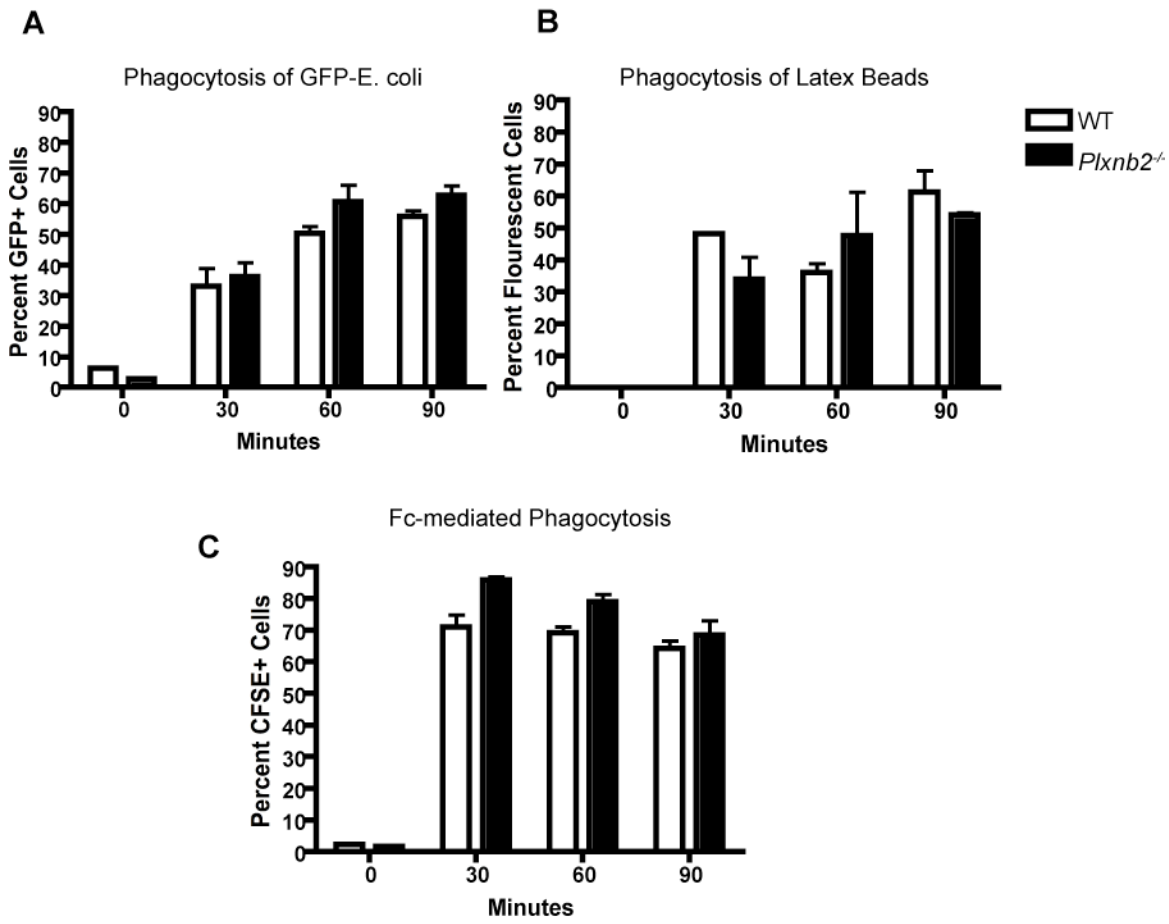
A
Phagocytosis of GFP-E. coli
Percent GFP+ Cells
0
10
20
30
40
50
60
70
80
90
0
30
60
90
Minutes
B
Phagocytosis of Latex Beads
Percent Flourescent Cells
0
10
20
30
40
50
60
70
80
90
0
30
60
90
Minutes
WT
Plxnb2-/-
C
Fc-mediated Phagocytosis
Percent CFSE+ Cells
0
10
20
30
40
50
60
70
80
90
0
30
60
90
Minutes

Figure 2.6

Figure 2.6: Plexin-B2 does not significantly affect phagocytosis.

A) Wild type and *Plxnb2*$^{-/-}$ bone marrow derived macrophages were incubated with GFP-E. coli, latex beads, or opsonized thymocytes (1:10 ratio E.coli, bead, or T cells: macrophage) for 30, 60, or 90 minutes. Uptake was measured by fluorescence and flow cytometry. Graphs are representative or four independent experiments. n=5 mice per group.

Chapter 3: Differential Expression of Plexin-B2 and Plexin-D1 in Dendritic Cell Maturation

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ABSTRACT

The plexin gene family is implicated in cellular movement and cell-cell communication during the course of the immune response. In this study, we characterize the expression and function of Plexin-B2 and Plexin-D1 in the immune system. We show that Plexin-B2 and Plexin-D1 are differentially expressed in dendritic cell (DC) populations. Expression of Plexin-B2 and Plexin-D1 is modulated upon activation of DCs by TLR ligands, TNFα, and anti-CD40. Absence of Plexin-B2 and Plexin-D1 in DCs does not affect the ability of these cells to upregulate costimulatory molecules or the ability of these cells to activate antigen specific T cells. Additionally, Plexin-B2 and Plexin-D1 are dispensable for chemokine-directed *in-vitro* migration of DCs towards CXCL12 and CCL19. However, absence of Plexin-B2 or Plexin-D1 on DCs leads to constitutive expression of IL-12/IL-23p40. Plexin-B2 and Plexin-D1 are negative regulators of IL-12/IL-23p40 signaling. This suggests that both Plexin-B2 and Plexin-D1 may play a role in dendritic cells in cytokine and T cell response.

INTRODUCTION

Semaphorins and plexins were initially identified as key molecules in axon guidance during neuronal development (Kolodkin et al. 1993; Winberg et al. 1998). Semaphorins are classified into three different groups based on their origin and structural homology; invertebrate, vertebrate and viral semaphorins (Mizui et al. 2009). Plexin receptors are divided into two large groups, invertebrate and vertebrate, and further subdivided into four different families, A-D (Takamatsu et al. 2010). Although plexins are considered receptors for the semaphorin ligands, this view has been revised as semaphorins are shown to also mediate signal transduction (Tamagnone et al. 1999; Castellani et al. 2002; Kruger et al. 2005; Yazdani et al. 2006). The interactions between semaphorins and plexins are promiscuous. Semaphorins can interact with multiple plexins on a single cell type or across multiple cell types and vice versa (Takamatsu et al. 2010). Plexins and semaphorins control cell movement and migration and have been implicated in neural cell function, vasculature formation, and organ development (van der Zwaag et al. 2002; Gu et al. 2005; Choi et al. 2008; Sakurai et al. 2010).

Recent work has implicated plexins and semaphorins in the immune system (Granziero et al. 2003; Wong et al. 2003; Walzer et al. 2005; Yamamoto et al. 2008). Several plexins and semaphorins are expressed by naïve and activated immune cells. Plexin-D1 and Semaphorin-3E are expressed in the thymus (Choi et al. 2008). Plexin-A1 and Semaphorin-6D are expressed on DCs and T cells (Wong et al. 2003; O'Connor et al. 2008). Semaphorin-4A is expressed by Th1 polarized T cells and DCs (Kumanogoh et al. 2002). Semaphorin-4D is expressed by T cells, DCs, and activated B cells (Delaire et al. 1998; Kumanogoh et al. 2000; Shi et al. 2000; Kumanogoh et al. 2002; Granziero et al. 2003;

Kumanogoh et al. 2005). Plexin-A4 is expressed by T cells, B cells and DCs (Yamamoto et al. 2008). Plexin-C1 is expressed by DCs (Walzer et al. 2005). The wide distribution of plexins and semaphorins across immune system cells and environments suggest that they function in immune system development and response.

The function of plexins and semaphorins on DCs has not yet been fully characterized. Plexin-A1 expression on DCs is required for proper T-cell activation and proliferation (Wong et al. 2003; Takamatsu et al. 2010). Semaphorin-6D, a known ligand for Plexin-A1, is expressed on activated T cells and is required for late-phase T cell proliferation (O'Connor et al. 2008). Mice deficient in Plexin-A4 develop exacerbated MOG-induced experimental autoimmune encephalomyelitis (EAE) (Yamamoto et al. 2008). Semaphorin-4D maintains B-cell homeostasis and facilitates humoral immune responses (Shi et al. 2000). Plexin and semaphorin function on DCs demonstrate their importance in the immune response.

To date, Plexin-D1 and Plexin-B2 research in the immune system has been limited. In other systems Plexin-D1 partners with two different semaphorin molecules: Semaphorin-3E and Semaphorin-4A (Gu et al. 2005; Toyofuku et al. 2007). Plexin-B2 has been found to have several semaphorin ligands including Semaphorin-3E, Semaphorin-4A, Semaphorin-4C, and Semaphorin-4D (Masuda et al. 2004; Lamont et al. 2009; Yukawa et al. 2010; Zielonka et al. 2010). Plexin-D1 was recently shown to be expressed by double positive thymocytes and facilitate their migration from the cortex into the medulla (Choi et al. 2008). Plexin-B2 is expressed on T-dependent germinal center B cells but not T-independent germinal center B cells, though the physiological consequence of this increase in expression are unknown (Yu et al. 2008). Studies of development in the model organism zebrafish have shown that Plexin-B2 and Plexin-D1 are antagonistic- Plexin-B2 deficiency results in

delayed migration of sprouting angioblast while Plexin-D1 deficiency results in early angioblast sprouting (Lamont et al. 2009). These findings show that Plexin-B2 and Plexin-D1 likley function in the same pathway but have differing roles. The expression of both of these plexins is important for timing of cell homing.

In this study, we report that Plexin-B2 and Plexin-D1 are differentially expressed in DCs. To address the role of Plexin-B2 and Plexin-D1 in DC development and function we utilized *Plxnd1*$^{-/-}$ *and Plxnb2*$^{-/-}$ animals. We used *in-vitro* and *in-vivo* approaches to examine the direct effect of Plexin-B2 and Plexin-D1 on DCs. We found that DCs lacking Plexin-B2 and Plexin-D1 were capable of inducing a normal T cell response in response to antigen. We also found that both *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are capable of migrating towards chemokines and are present in the spleen in patterns comparable to wildtype. *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are capable of secreting normal amounts of TNFα and IL-6. However, both *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are hyper-responsive in their secretion of IL-12/ IL-23p40. Our results show that in DCs, both Plexin-B2 and Plexin-D1 are differentially expressed and the absence of either does not impact T cell proliferation response, chemokine directed migration, and IL-6 and TNF secretion. However, both Plexin-B2 and Plexin-D1 are negative regulators of IL-12/IL-23p40 response, demonstrating that they may function in DCs in the same pathway and that this pathway may be modulated by their differential expression.

RESULTS

Plexin-B2, Plexin-D1, and Semaphorin-3E Expression in Immune Cells

Expression patterns of individual plexins in the immune system during cell maturation or activation have been reported in the literature. Plexin-B2 is expressed on B cells from T cell dependent germinal centers but not T independent germinal centers (Yu et al. 2008). Plexin-D1 is expressed by thymocytes and further downregulated with T cell maturation (Choi et al. 2008). We extended Plexin-B2 and Plexin-D1 expression studies to include another immune cell type, DCs. DCs are required for T cell priming in the secondary lymphoid organs. We observed Plexin-B2 and Plexin-D1 expression in sorted splenic myeloid DCs (mDCs) ($CD11b^{+}CD11c^{+}$) (Fig. 3.1A, B, and C). Plexin-B2 is expressed at day 6 of maturation of bone marrow derived DCs treated with GM-CSF and IL-4, then decreases mid-maturation at day 8, and then increases at maturation day 10 and treatment with TNFα and IL-6 (Figure 1A). Plexin-B2 expression is not increased by treatment with toll-like receptor (TLR) ligands (P3C, TLR1/2), lipopolysaccharide (LPS, TLR4), or CpG (TLR9). Plexin-B2 is highly expressed by plasmacytoid DCs ($CD11c^{+}B220^{+}mPDCA1^{+}$) (Fig. 3.1A, B).

The expression pattern of Plexin-B2 is in contrast with the expression pattern of Plexin-D1, which increases throughout the maturation of bone marrow-derived DCs (BMDCs) and with treatment by TLR ligands P(3)C, LPS, and CpG (Fig. 3.1C). Plexin-D1 expression is not increased in response to treatment with TNFα or CD40L, and is not expressed on plasmacytoid DCs. Both Plexin-B2 and Plexin-D1 are expressed on splenic *ex-vivo* DCs (Fig. 3.1A, C).

We confirmed the expression of Plexin-B2 throughout DC maturation at the protein level using a monoclonal antibody against Plexin-B2 (Fig. 3.1B). In conventional DCs that are produced with GM-CSF and IL-4, our data showed that expression of Plexin-B2 is

bimodal throughout maturation. In plasmacytoid DCs that are matured with Flt3L Plexin-B2 is highly expression throughout maturation. These data support the differential cDNA expression pattern of Plexin-B2 (Fig. 3.1A).

Studies have shown that the predominant Plexin-D1 partner in the immune system is Semaphorin-3E, although the specific cell type providing the ligand is unknown (Gu et al. 2005; Choi et al. 2008). These studies show that Semaphorin-3E is expressed in the thymic medulla where it creates a gradient that is responsible for migration of Plexin-D1 expressing thymocytes from the cortex into the medulla (Choi et al. 2008). The immune system binding partner for Plexin-B2 is unknown. However, in zebrafish angioblast, Semaphorin-3E is the ligand for Plexin-B2, and Plexin-D1 antagonizes the Plexin-B2/Semaphorin-3E pathway. Upon observing the opposing expression patterns of Plexin-B2 and Plexin-D1 in cDCs, we hypothesized that Semaphorin-3E may also be present on DCs. We analyzed Semaphorin-3E expression in a number of immune cells (Figure 1D). Our data show that Semaphorin-3E is minimally detected in naïve and activated T and B cell populations. However, Semaphorin-3E is highly expressed on Th2 skewed T cells and splenic cDCs. The expression pattern of Semaphorin-3E suggests that partnering of this protein with Plexin-B2 and Plexin-D1 during the course of an immune response may be important for T cell activation.

Plxnb2$^{-/-}$ and Plxnd1$^{-/-}$ DCs stimulate T cells similarly to wild type

DCs are proficient antigen presenting cells required for proper T cell selection in the thymus and activation of naïve T cells in the periphery (Cella et al. 1997; Hanahan 1998). DCs take up antigen and present it to T cells in the context of MHC molecules (Hanahan 1998). Upon cognate antigen encounter, T cells proliferate, release a series of cytokines and

function as cytotoxic T-lymphocytes or helper T cells (Th) (Hanahan 1998). To further define the role of Plexin-B2 and Plexin-D1 in DCs, we performed an *in-vitro* antigen presentation assay using transgenic T cells specific for ovalbumin (OVA), OTII T cells. Given that *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice die shortly after birth we created fetal liver chimeric mice to study Plexin-B2 and Plexin-D1 in the immune system. Wild type congenic mice were reconstituted with hematopoietic cells from E14 fetal livers of Plxnb2$^{-/-}$ or *Plxnd1*$^{-/-}$ mice (Fig. 3.2).

We first determined the ability of *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs to take up OVA-protein. DCs were cultured for 2 hours in the presence of FITC-labeled OVA protein and the amount of OVA taken up by DCs was assessed by flow cytometry. *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$ and wild type DCs take up antigen equivalently as shown by the level of mean fluorescence intensity of the analyzed DCs (Fig. 3.3A). To determine the ability of the DCs to stimulate T cells, freshly isolated carboxyfluorescein succinimidyl ester (CFSE)-labeled OTII T cells were cultured in the presence of OVA protein-pulsed *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type splenic DCs for three days. Non OVA-pulsed DCs were used as a negative control. Proliferation of Vb5^{+} OTII T cells was assessed by flow cytometry. As shown in Figure 3.3B, *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are capable of stimulating T cells similarly to wild type. These data suggest that both Plexin-B2 and Plexin-D1 are dispensable during *in-vitro* activation of CD4^{+} T cells by DCs.

Plexin-B2 and Plexin-D1 do not affect the migration of DCs

Plexins and semaphorins have been implicated in migration of many different cell types including neuronal, endothelial, and immune cells (Kruger et al. 2005). In zebrafish angioblast cells, knockdown of Plexin-B2 or its ligand Semaphorin-3E yields delayed

sprouting of intersegmental (ISV) angioblast during development (Lamont et al. 2009). Knockdown of Plexin-D1 in zebrafish embryos results in an opposite effect of early ISV sprouting (Lamont et al. 2009). In the mouse nervous system, *Plxnb2*$^{-/-}$ animals show defects in neuronal cell homing that result in neural tube closure defects and cerebellum disorganization (Friedel et al. 2007). Plexin-D1 is required for endothelial cell patterning (Gu et al. 2005) as well as migration of DP thymocytes from the cortex into the medulla during thymic maturation (Choi et al. 2008). Therefore we investigated the ability of *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs to migrate towards chemokines that are associated with lymph node homing CXCL12 and CCL19 (Takamatsu et al. 2010). Transwell assays of DCs and CXCL12 (Fig. 3.4A), CCL19 (Fig. 3.4B) show that both *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs migrate similarly to wild type. To further investigate the migration and homing capabilities of DCs deficient in Plexin-B2 or Plexin-D1, we visualized macrophages, DCs, and B cells in the spleens of *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type reconstituted mice. In Fig. 3.4C, we found that CD11b^{+} macrophages, CD11c^{+} DCs, and B220^{+} B cells were present in similar architecture in *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type spleens. Our data show that Plexin-B2 and Plexin-D1 do not affect migration towards lymph node homing cytokines or architecture of DCs, macrophages, or B cells to the spleen.

Plxnb2$^{-/-}$ *and Plxnd1*$^{-/-}$ *DCs are negative regulators of IL-12/IL-23p40.*

To further assess development of *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs, we stimulated BMDCs from *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type animals with LPS. We determined expression of co-stimulatory molecules on the surface of DCs. Surface levels of CD40, CD80, CD86, and I-

Ab were equivalent between wild type, *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice at both the basal level and post activation.

To characterize DC activation we performed ELISAs of the common cytokines that are associated with DC function including IL-6, TNFα, and IL-12/IL-23p40. Supernatants were collected from treatment of DC cultures that were treated with LPS, P3C, and anti-CD40. As shown in Figure 3.5A, we determined that levels of IL-6 and TNFα that were released in the culture supernatants were equivalent between *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type DCs. However, we observed that levels of IL-12/IL-23p40 were higher in both *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs compared to wild type levels in CD40L stimulated, LPS treated, or unstimulated DCs.

Taken together, these data suggest that despite expression of Plexin-B2 and Plexin-D1 by the DC population and their upregulation post activation, these proteins are not required for expression of costimulatory molecules on the surface of DCs post activation or for production of IL-6 and TNFα. Instead, Plexin-B2 and Plexin-D1 are required for the negative regulation of IL-12/IL-23p40 by DCs.

DISCUSSION

In this study, we characterize the expression of Plexin-B2, Plexin-D1, and Semaphorin-3E in various immune cell types. We report that Plexin-B2 is expressed early in development of BMDCs, decreases, and then increases at maturation and with treatment of TNFα or LPS but not TLR ligands. Plexin-B2 is highly expressed by pDCs. The bi-modal expression pattern in cDCs and high expression in pDCs of Plexin-B2 is opposite that of Plexin-D1. Plexin-D1 expression increases in myeloid DCs throughout maturation, and is

not expressed on pDCs. Expression of Plexin-D1 by myeloid DCs is increased when these cells are activated in the presence of TLR agonists. Expression studies of a ligand for Plexin-B2 and Plexin-D1, Semaphorin-3E, revealed that Semaphorin-3E is highly expressed by Th2-type T cells and DCs. Based on differential expression of Plexin-B2 and Plexin-D1 on DCs and previous studies demonstrating a role for Plexin-D1 in thymocyte development (Choi et al. 2008), we hypothesized that absence of Plexin-B2 and/or Plexin-D1 would lead to abnormal T cell-DC interactions.

Although a role for Plexin-D1 in the thymocyte migration has been previously reported, these studies do not address the role of Plexin-D1 in T cell activation during immune responses (Choi et al. 2008). Studies of other plexins have demonstrated that plexins can have a profound impact on T cell-DC interactions. For example, Plexin-A1 deficient DCs result in an 85% reduction of T cell proliferation in response to antigen both *in-vitro* and *in-vivo* (Wong et al. 2003; O'Connor et al. 2008). Our analysis of *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice did not reveal a role for Plexin-B2 or Plexin-D1 in antigen uptake by DCs or transgenic T cell proliferation in response to antigen. These findings suggest that while other plexins are required for T cell proliferation, Plexin-B2 and Plexin-D1 likely participate in other functions of DCs.

Plexins and semaphorins mediate cell migration in the immune system. Plexin-C1 is expressed on DCs and facilitates inhibition of chemokine induced migration when bound to ligand (Walzer et al. 2005). Plexin-A1 is required for transmigration of DCs across lymphatic endothelial cells yet is dispensable for chemokine induced migration in-vitro (Takamatsu et al. 2010). DCs migrate towards the lymph node homing chemokines CXCL12 and CCL19 upon maturation. Our studies show that Plexin-B2 and Plexin-D1 do not play a

role in migration towards lymph node homing cytokines. We also show that in steady state DC cell number and pattern in the spleen are similar between *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type. DCs are very dynamic in their migration patterns throughout maturation and activation *in-vivo,* and this cannot necessarily be mimicked *in-vitro*. Future studies should address a role for migration of Plexin-B2 and Plexin-D1 *in-vivo*. Studies *in-vivo* may reveal that Plexin-B2 and Plexin-D1 are involved in migration under specific conditions such as activation by pathogen or in specific immune environments.

To further investigate the roles of Plexin-B2 and Plexin-D1 in DC activation, we assayed cell surface markers and cytokine production by *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs. Our data show that Plexin-B2 and Plexin-D1 are not required for upregulation of activation markers CD40, CD80, CD86, or I-Ab in response to LPS induced activation. However, in untreated conditions and in response to LPS both *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs show increased levels of IL-12/IL-23p40. Levels of TNFα and IL-6 are not affected by Plexin-B2 or Plexin-D1, revealing that this effect is specific for IL-12/IL-23p40. Future studies should address if both IL-12 and IL-23, which share the common p40 subunit, are affected by both Plexin-B2 and Plexin-D1. Downstream physiological consequences of overproduction of IL-12/23p40 in *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice, including Th skewing, response to pathogen, and potential pathways that mediate this effect should be assessed.

In summary, our studies reveal that Plexin-B2 and Plexin-D1 are differentially expressed in DCs, yet both mediate negative regulation of IL-12/IL-23p40. These findings suggest crosstalk between the signaling pathways of Plexin-B2 and Plexin-D1 in DCs, similar to that previously reported in zebrafish angioblast (Lamont et al. 2009). The data suggest Plexin-B2 and Plexin-D1 function at different times of development of the DC and

may also function in different environments or under different cellular conditions. The differential expression of Plexin-B2 and Plexin-D1 demonstrate that control of cell processes by plexins may be determined by their expression.

MATERIALS AND METHODS

Mice

C57BL/6 and congenic C57BL/6 CD45.1 mice were obtained from the National Cancer Institute (Boston, MA). *Plxnd1*$^{+/-}$ mice were a gift from Dr. Thomas Jessell's laboratory and have been described (Gu et al. 2005). *Plxnb2*$^{+/-}$ mice were a gift from Dr. M. Tessier-Lavigne and have been described (Friedel et al. 2007). *Plxnb2*$^{+/-}$ and *Plxnd1*$^{+/-}$ mice were backcrossed in house at least 10 generations. OT-II mice (B6.Cg.Tg(TcraTcrb)425Cbn/J), specific for the ovalbumin residue 323-339, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility at University of North Carolina. These studies were approved by the University of North Carolina Animal Care and Use Committee. For fetal liver chimeras *Plxnd1*$^{+/-}$ or *Plxnb2*$^{+/-}$ mice were crossed for over 10 generations with C57BL/6 mice and intercrossed to obtain $^{-/-}$ and $^{+/+}$ embryos. Fetal livers were prepared from E14 embryos post PCR genotyping as previously described (Gu, Yoshida et al. 2005). Fetal liver cells were injected (iv) into lethally irradiated C57BL/6 CD45.1 mice. Mice were analyzed 6-10 weeks post reconstitution. Mice were allowed to reconstitute for 6-8 weeks before use.

ELISA

Splenic DCs were isolated from wild type, *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ animals and were

stimulated for 24 hours in the presence Pam3Cys or LPS. The culture supernatants were tested for IL6, TNFα, and IL-12/IL-23p40 cytokine levels by ELISA (Ebioscience, San Diego, CA).

Antibodies and FACS

Monoclonal Abs included: B220 (RA3-6B2), CD23 (B3B4) and APC-Alexa750-conjugated streptavidin from BD Pharmingen (San Diego, CA); CD45.2 (104), CD4 (L3T4), CD8 (Ly-2), IFNgXMG1.2), IL2 (JES6-5H4), IL4 (11B11), CD3 (145-2C11), CD28 (37.51), CD62L (MEL-14), GL7 (Ly-77), CD11b (M1/70), CD11c (N418), CD21 (eBio8D9) and TCRβ (H57-597) from eBioscience (San Diego, CA). Secondary antibodies included anti-FITC-Alexa488 and Alexa405-conjugated streptavidin from Invitrogen (Carlsbad, California). Single cell suspensions of different tissues were counted and 10^6 cells were suspended in FACS buffer (1xPBS plus 2% FBS) and stained with various antibody combinations. All flow cytometry was performed on a FACSCalibur and analyzed with FlowJo software (Tree Star).

RT-PCR and Quantitative RT-PCR analysis

RNA was isolated from tissues or sorted resting T cell populations ($CD62L^{hi}$ $CD4^+$ T cells, $CD62L^{hi}CD8^+$ T cells) as well as Th0, Th1 and Th2 cells using a Qiagen RNA extraction kit. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Primers used for RT-PCR and real-time PCR analysis were: HPRT, 5'-GCTGGTGAAAAGGACCTCT-3', 5'-CACAGGACTAGAACA CCTGC-3'; *Plxnb2* 5'- CTAGACATCCCTGAGTCACG-3', 5'- AGTCAGCAGTGATGCAAAGT-3'; *Plxnd1*, 5'-CCTGGGTCACCTCTGTGTTT-3', 5'-

TATCTGTCAGGCAGGGGTTC-3'; and *Semaphorin3E*, 5'-AGGCCCTGAATACCACTGGTC-3', 5'-GGTTCCTGTGCCAGCAAAGT-3'. Quantitative real-time PCR was performed using SYBR Green reagent in a BIORAD iCycler.

Cell culture

T cell activation: Th1 vs. Th2 skewing was conducted as follows. Sorted $CD62L^{hi}$ $CD4^{+}$ naive splenic T cells ($2x10^6$ cells/ml) were cultured for 3 days with immobilized anti-CD3 (5mg/ml) and anti-CD28 (5mg/ml) in the presence of anti-IL4 (10mg/ml) and IL-12 (10ng/ml) (Th1-skewing conditions) or in the presence of anti-IFNγ (10mg/ml) and IL-4 (10ng/ml) (Th2-skewing conditions. *DC culture*: Murine bone marrow DCs were isolated from wild type, *Plxnb2*$^{-/-}$, or *Plxnd1*$^{-/-}$ mice and were cultured in the presence of GM-CSF and IL-4 as previously described (van Deventer et al. 2002). *T cell culture:* T cells from OT-II mice and purified by negative selection (STEMCELL). *DC:T cell co-cultures:* DCs were harvested at day 10 and pulsed overnight with 50mg/ml OVA (Sigma-Aldrich). 200,000 DCs were then washed and cultured in a 1:10 ratio with T cells from OT-II transgenic T cells in 6 well plates.

Histology

Spleens of naïve *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice were embedded in OCT compound, snap frozen, and stored at -80°C. 5 mm sections were prepared and fixed with 1:1 Acetone:Methanol for 10 min at -20°C and labeled with various combinations of fluorescently labeled CD11b, CD11c, TCRb and B220 mAb. FITC signal was amplified

using anti-FITC-Alexa488 mAb. Streptavidin-AlexaFluor405 was used to amplify B220-biotin signal (blue). Images were acquired using a Zeiss LSM 710 confocal immunofluorescent microscope.

Statistical analysis

Statistical significance was determined with two-tailed Student's t test or analysis of variance (ANOVA). All p values less than 0.05 were considered significant.

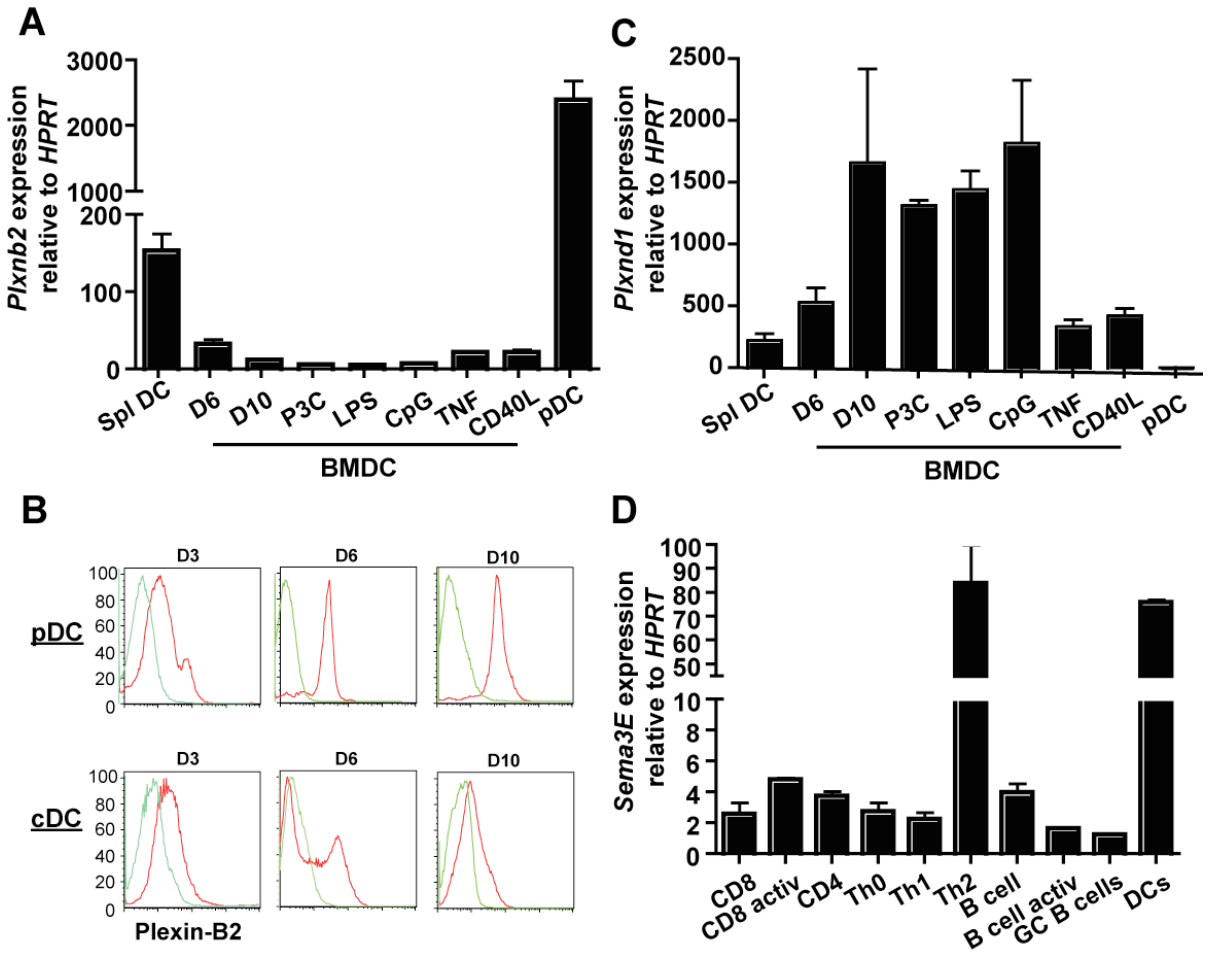
A
Plxnb2 expression relative to HPRT
3000
2000
1000
200
100
0
Spl DC
D6
D10
P3C
LPS
CpG
TNF
CD40L
pDC
BMDC
C
Plxnd1 expression relative to HPRT
2500
2000
1500
1000
500
0
Spl DC
D6
D10
P3C
LPS
CpG
TNF
CD40L
pDC
BMDC
B
D3
D6
D10
pDC
cDC
100
80
60
40
20
0
Plexin-B2
D
Sema3E expression relative to HPRT
100
90
80
70
60
50
10
8
6
4
2
0
CD8
CD8 activ
CD4
Th0
Th1
Th2
B cell
B cell activ
GC B cells
DCs

Figure 3.1

Figure 3.1. Plexin-B2, Plexin-D1, and Semaphorin-3E expression.

(A) Expression of *Plxnb2* in splenic DCs, BMDCs at day 6 (D6) and day 10 (D10), D10 post 16 hour activation by TLR ligands P3C (1 ug/ml), (LPS (1 ug/ml), CpG (4 ug/ml), TNF (20 ng/ml), CD40L (1 ug/ml), and plasmacytoid DCs (pDCs) as measured by real-time PCR. Data are representative of three independent experiments. (B) Expression of *PlxnB2* in BM-derived pDCs and cDCs at D3, D6, and D10. Green lines indicate IgG control antibody staining, red histograms are *Plxnb2* antibody staining. (C) Expression of *Plxnd1* in sDCs, BM-derived DCs D6, D10, post activation, and pDC as measured by real-time PCR. (D) Expression of *Sema3E* in sorted naïve and activated T cell and B cell populations, and DCs. Data are representative of 3 independent experiments.

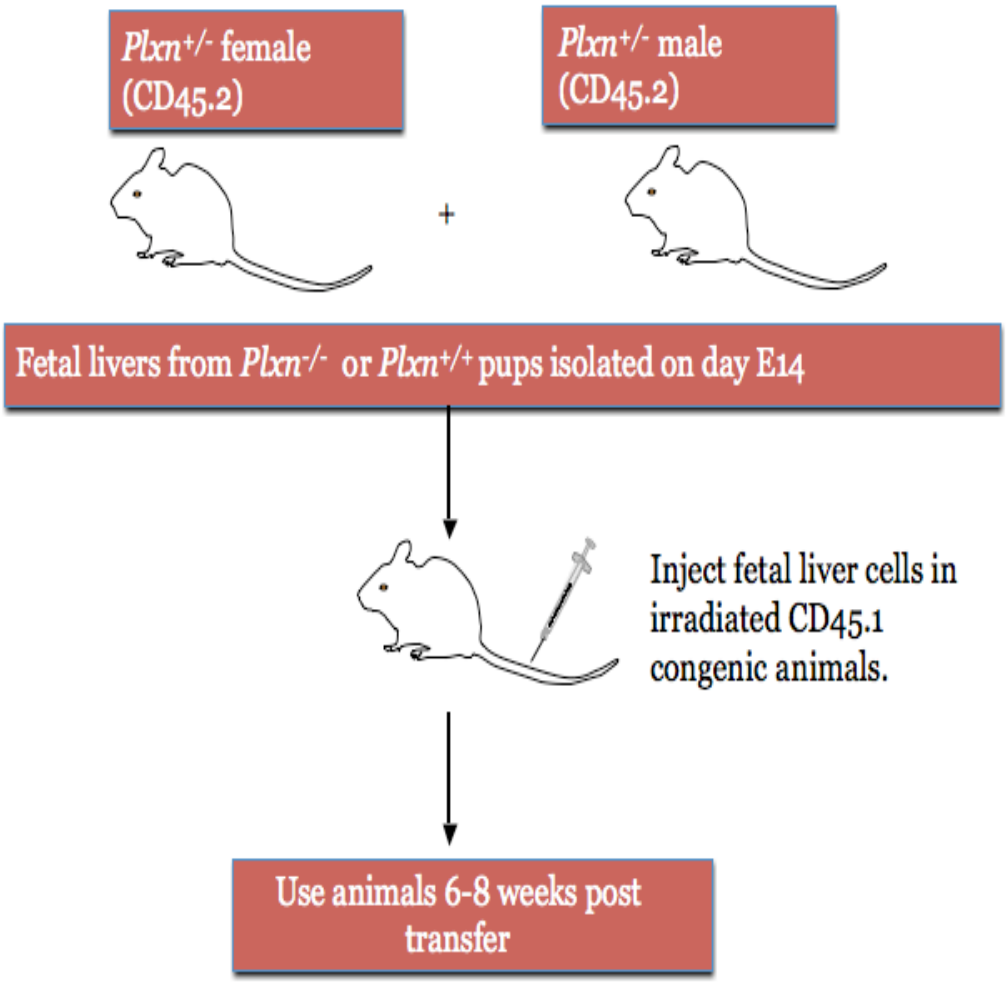
Plxn+/- female (CD45.2)
Plxn+/- male (CD45.2)
+
Fetal livers from Plxn-/- or Plxn+/+ pups isolated on day E14
Inject fetal liver cells in irradiated CD45.1 congenic animals.
Use animals 6-8 weeks post transfer

Figure 3.2

Figure 3.2. Schematic of fetal liver transplant.

Plxnb2$^{+/-}$ or *Plxnd1*$^{+/-}$ mice are crossed and fetal livers of wild type or *Plxnb2*$^{-/-}$ or *Plxnd1*$^{-/-}$ embryos are harvested at day 14 of gestation. Fetal liver cells are harvested by homgenization. 2 x 10^6 fetal liver cells are then transplanted into lethally irradiated congenic CD45.1+ mice by tail vein injection. Animals reconstitute the immune system with donor cells for six to eight weeks post transplant.

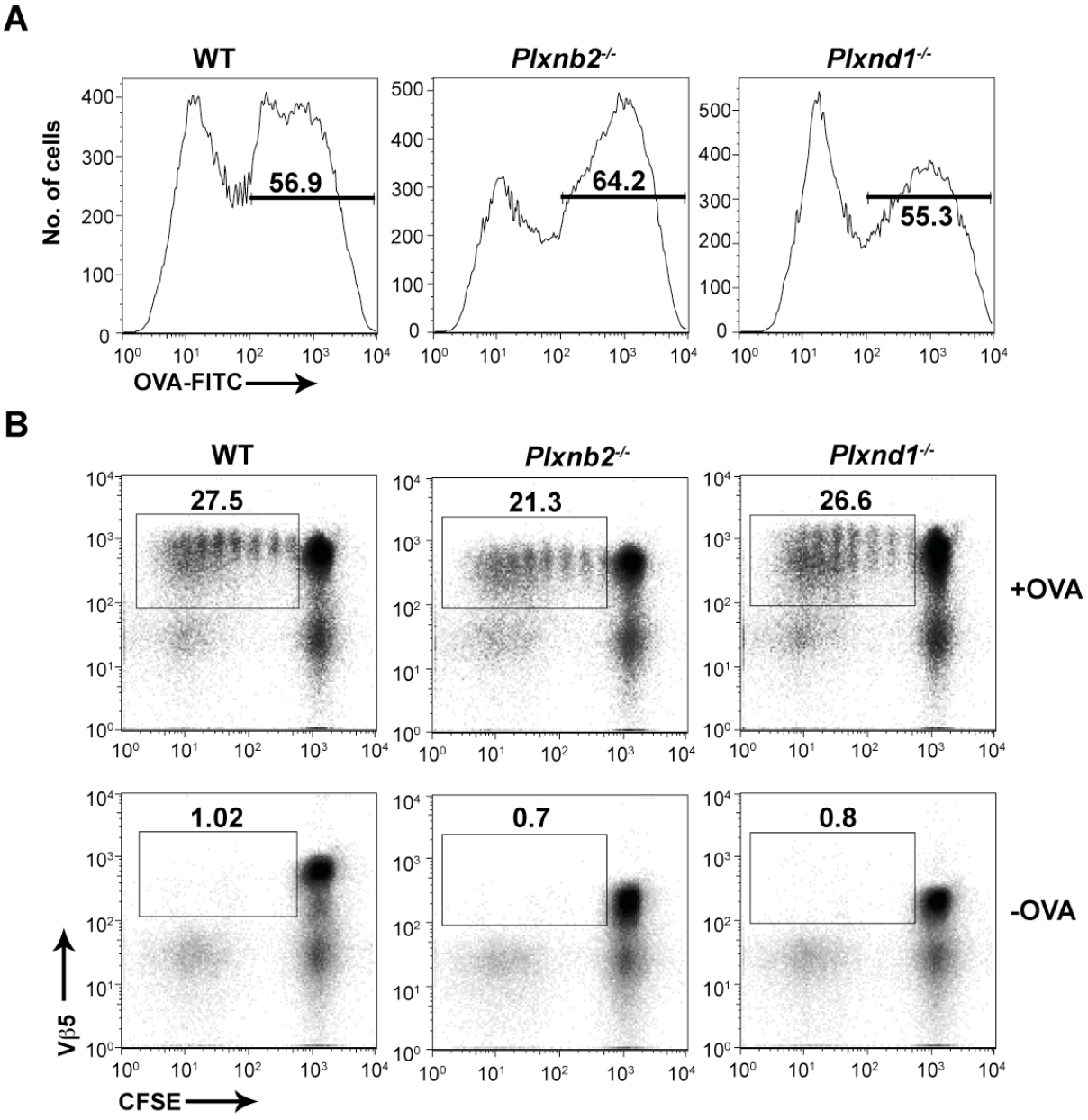
A
WT
Plxnb2-/-
Plxnd1-/-
No. of cells
56.9
64.2
55.3
OVA-FITC
B
WT
Plxnb2-/-
Plxnd1-/-
27.5
21.3
26.6
+OVA
1.02
0.7
0.8
-OVA
Vβ5
CFSE

Figure 3.3

Figure 3.3. *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs efficiently stimulate antigen specific T cells. (A) OVA uptake for *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type DCs. DCs were isolated from spleens of mice reconstituted with *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type fetal liver cells and cultured in the presence of OVA-FITC for 2 hours. OVA uptake was assessed by flow cytometry. Data are representative of 2 independent experiments. n=6 mice per group. (B) DCs were isolated from spleens of mice reconstituted with *Plxnb2*$^{-/-}$, *Plxnd1*$^{-/-}$, and wild type fetal liver cells. DCs were then co-cultured with OTII-specific T cells in the presence of OVA and T cell proliferation was assessed by CFSE dilution 72 hours later using flow cytometry. Data are representative of 3 independent experiments. n=9 mice per group.

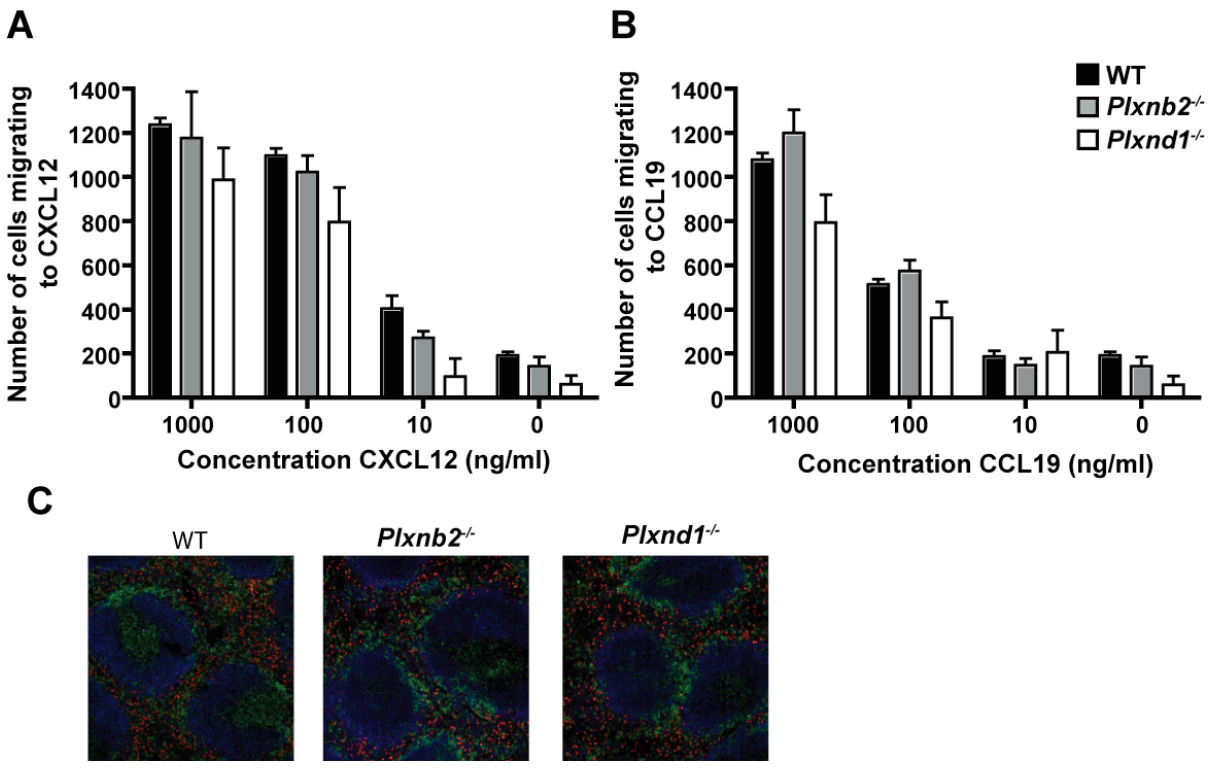
A
Number of cells migrating to CXCL12
1400
1200
1000
800
600
400
200
0
1000
100
10
0
Concentration CXCL12 (ng/ml)
B
Number of cells migrating to CCL19
WT
Plxnb2-/-
Plxnd1-/-
1400
1200
1000
800
600
400
200
0
1000
100
10
0
Concentration CCL19 (ng/ml)
C
WT
Plxnb2-/-
Plxnd1-/-

Figure 3.4

Figure 3.4. *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs migrate similarly to WT control towards chemokine gradients.

(A) Purified wildtype (black bar), *Plxnb2*$^{-/-}$ (grey bar), and *Plxnd1*$^{-/-}$ (open bar) DCs were placed in upper wells and subjected to *in-vitro* migration assays in the presence of medium alone, CXCL12 and CCL19. Migrated cells were quantified by toxilight (Lonza, Basel, Switzerland) according to the manufacturers instructions and normalized to a standard curve n=6-7 mice per group. (B) Five mm sections of spleens from wildtype, *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ mice were labeled with B220-AF350 (blue), CD11b-PE (red) and CD11c-FITC (green). FITC signal was amplified using anti-FITC-AF488. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. n=3 mice per group.

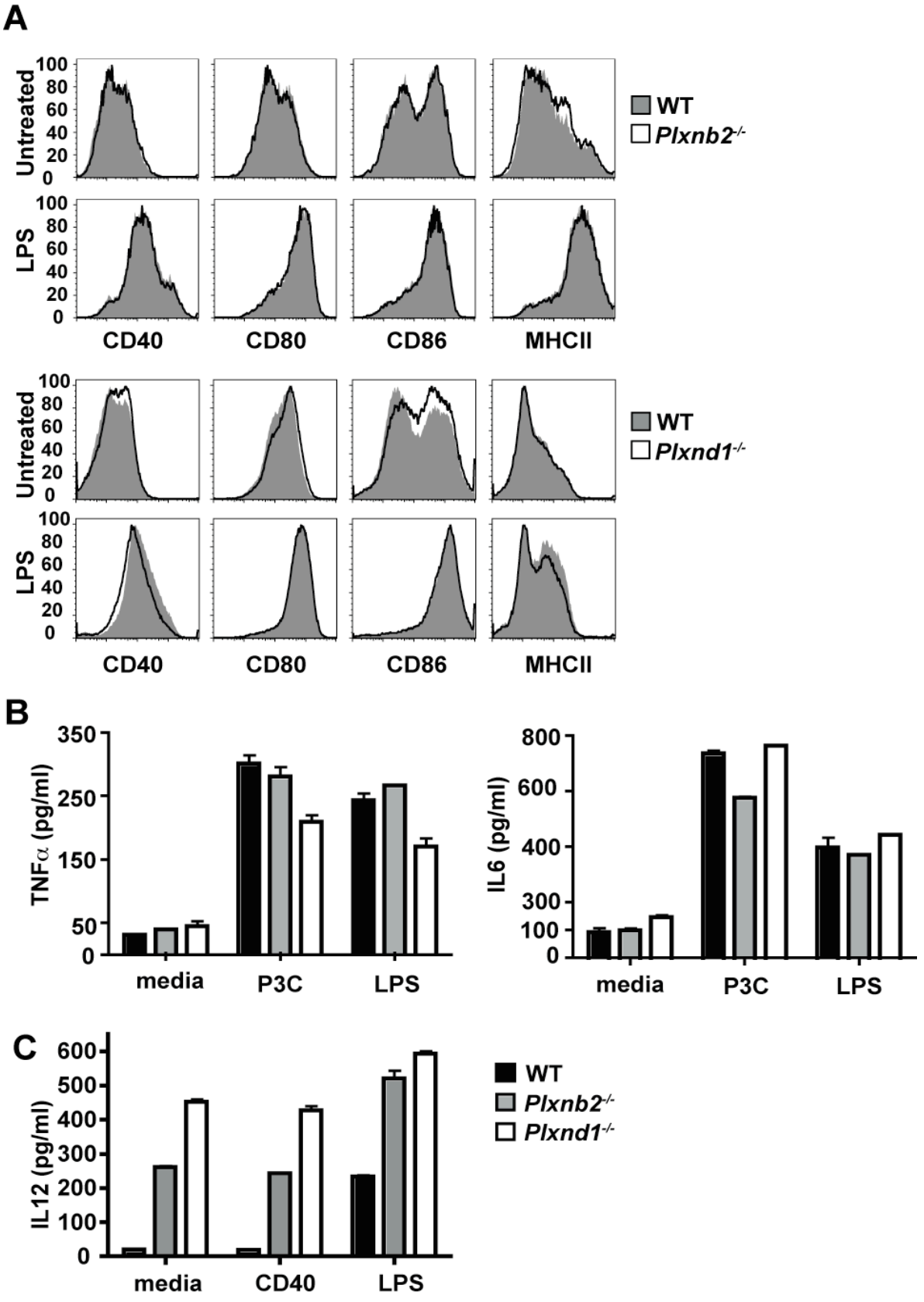
A
Untreated
LPS
100
80
60
40
20
0
CD40
CD80
CD86
MHCII
WT
Plxnb2-/-
Plxnd1-/-
B
TNFα (pg/ml)
350
250
150
50
0
media
P3C
LPS
IL6 (pg/ml)
800
600
400
300
100
C
IL12 (pg/ml)
600
500
400
300
200
100
media
CD40
LPS

Figure 3.5

Figure 3.5. *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs overproduce IL-12/IL-23p40.

(A) *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are able to upregulate cell surface receptors. DCs were derived in the presence of GM-CSF and IL4 from the bone marrow of mice reconstituted with *Plxnb2-/-, Plxnd1*$^{-/-}$, and wild type fetal liver cells. DCs were then cultured in the presence of LPS and cell surface receptor expression was assessed 24 hours later using flow cytometry. Data are representative of 3 independent experiments. n=6 mice per group. (B) *Plxnb2*$^{-/-}$ and *Plxnd1*$^{-/-}$ DCs are able to produce inflammatory cytokines in response to TLR stimuli and anti-CD40. DCs were cultured in the presence of LPS and anti-CD40 for 24 hours. Culture supernatants were assessed for cytokine production by ELISA. Data are representative of 3 independent experiments. n=3 - 4 mice per group.

Chapter 4: Conclusions and Future Directions

Plexin-B2 Expression in the Immune System

Data in Chapters 2 and 3 show that Plexin-B2 is expressed in macrophages, cDCs, and pDCs. This is the first report of Plexin-B2 expression in defined populations of the innate immune system. Our data show that Plexin-B2 is highly expressed on both peritoneal and bone marrow-derived macrophages yet its expression is not modulated by stimulation with TLR agonist to activate the macrophages. This expression pattern suggests that Plexin-B2 is active throughout the life cycle of the dendritic cell and may mediate functions during quiescence as well as cellular activation. Macrophages also express Plexins-A1- A3 (Ji et al. 2009). In contrast to Plexin-B2, the expression level of Plexins-A1-A3 increases throughout M-CSF stimulated maturation. M-CSF stimulated maturation yields an M2, alternatively activated, less inflammatory type of macrophage. The increase in Plexin-A1-A3 expression suggests function during M-CSF maturation that might be less inflammatory and more related to the modulation of adaptive immunity. As an example, Plexin-A1 expression is reduced upon activation of macrophages with LPS, which results in a more inflammatory, classically activated M1 type of macrophage (Judas et al. 2003; Ji et al. 2009). We and others have indeed confirmed that a major function of Plexin-A1 is in T cell activation. This expression pattern is in contrast to Plexin-A4, which is high on both immature and M-CSF matured macrophages, suggesting a more constant level of function (Ji et al. 2009) . Thus, modulation of plexin expression in macrophages may serve as a method of regulating plexin signaling throughout cell maturation and activation. Studies of Plexin-B2 should compare its

expression pattern in macrophages to an exhaustive study of expression of other plexins in macrophages throughout maturation and activation. This data would yield a more complete understanding of plexins that are active at specific stages in macrophage maturation, polarization, or activation state. Additional studies addressing the expression of plexins in macrophages from different sites in the body and during conditions such as tumorgenesis, angiogenesis, and immune response may also provide important clues as to the function of plexins in macrophages.

In contrast to macrophages, Plexin-B2 expression is modulated throughout the life cycle of the BM-derived cDC. In cDCs derived from cultures of bone marrow with GM-CSF and IL-4, Plexin-B2 is expressed in a bi-phasic fashion. It is expressed early in maturation, is reduced at mid-maturation, and then is slightly upregulated at maturation. In pDCs derived from FLT3L supplemented bone marrow cultures, expression of Plexin-B2 is upregulated in the early stages of maturation and continues to remain at high levels throughout maturation. The pDC is most similar to macrophages in expression pattern in that Plexin-B2 expression remains unabated with maturation. These data suggest that Plexin-B2 might be functionally active during the lifespan of macrophages and pDCs, but its function might be more specific to the early and late maturation stages of cDC. However, in *ex-vivo* splenic cDCs, Plexin-B2 is more highly expressed, suggesting that culture conditions may alter plexin expression in cells, and this must be carefully considered during experimental design.

Plexin-A1 is one of the better studied plexins in the immune system. It is expressed on cDCs and is modulated throughout maturation. Plexin-A1 levels are very low at the early and mid stages of maturation, and quickly spike at later stages and with TNFα (Wong et al. 2003). Plexin-A1 is not expressed in macrophages (Wong et al. 2003) nor pDCs (Eun,

unpublished observation), suggesting a cDC specific function. Intriguingly, one of the ligands for Plexin-A1, Sema6D, does not appear on T cells until the later stages of cell proliferation that occurs in response to antigen presentation by the DC (O'Connor et al. 2008). Without Plexin-A1 T cell proliferation is stopped at late stage (O'Connor et al. 2008). Plexin-A1 is also required for entry of DCs into the lymphatic system through another ligand, Sema3A (Takamatsu et al. 2010). This example highlights how timing of expression of both plexins and their ligands are critical in controlling their signaling responses.

Plexin-B2 in Immune System Development

Plexins and their ligands are important in development of the nervous and vascular systems. For example, Plexin-A2 and its ligand Nueropilin-2 mediate cell migration and cell homing during development at the interface of the peripheral and central nervous system (Chauvet et al. 2008). Deficiency of Plexin-D1 or its vascular system ligand Semaphorin-3A result in embryonic lethality in the mouse due to defects in vascular patterning (Gitler et al. 2004; Gu et al. 2005). Plexin-B2 is critical during embryogenesis in the nervous system (Friedel et al. 2007). *Plxnb2*$^{-/-}$ mice display severe neural tube closure defects that result in exencephaly and embryonic lethality (Friedel et al. 2007; Hirschberg et al. 2010). The defects in neural tube closure are attributed to defects in cell migration and homing (Friedel et al. 2007). Based on the expression of Plexin-B2 on innate immune cells, we hypothesized that Plexin-B2 may have an effect on immune system development.

To study the formation of the immune system in embryonic lethal mice, we created fetal liver chimeric mice from wild type and *Plxnb2*$^{-/-}$ fetal livers. As discussed in Chapter 2, the immune system of *Plxnb2*$^{-/-}$ mice is reconstituted normally in both the splenic and bone

marrow compartments. Fetal liver transplants into Ly5.2^{+} mice reveal that percent of B220^{+} B cells, CD11c^{+} cDCs, PDCA-1^{+} / B220^{+} pDCs, and F4/80^{+} macrophages found in spleen are equivalent between wild type and *Plxnb2*$^{-/-}$ reconstituted mice. T cell and NK cell development is normal, as TCR^{+}CD4^{+}, TCR^{+}CD8^{+}, NK1.1^{+}CD4^{+} NKT cells, and NK1.1^{+} cell percentages are equivalent. The data in Chapter 3 show that the architecture of macrophages, dendritic cells, and B cells of *Plxnb2*$^{-/-}$ spleens are normal compared to wild type. The finding that immune system development and homing are normal in the *Plxnb2*$^{-/-}$ mouse is surprising, given the extreme defects found in the nervous system of these mice. It is possible that non-immune expression of Plexin-B2 in the recipient mouse is enough to mask any Plexin-B2 related defects in immune development and homing. It is also possible that under different conditions of pathologic or beneficial immune activation, such as development of autoimmunity, cancer, or immune response to pathogen, a defect in *Plxnb2*$^{-/-}$ immune cell homing or proliferation could be observed. Future Plexin-B2 studies should address more functional aspects of DCs and macrophages, as well as immune system development in response to stimulation and pathogens.

Small GTPases and Plexin-B2

As described in Chapter 2, Plexin-B2 is a negative regulator of the GTPases, Rac and Cdc42, but not Rho in macrophages. Extensive studies of the closely related Plexin-B1 have demonstrated that Plexin-B1 is a critical regulator of not only Rac and Rho, but also M-Ras and R-Ras GTPases (Driessens et al. 2002; Vikis et al. 2002; Oinuma et al. 2004; Saito et al. 2009). Plexin-B1 regulation of Rho has also been shown to require Rnd1 GTPase (Oinuma et al. 2003). The functional outcome of the Plexin-B1 regulation of the Rho family of

GTPases is very finely regulated modulation of the actin cytoskeleton and cell motility. Other plexins have also been found to mediate Rho family GTPase signaling. Plexin-A1 modulates antigen specific T cell proliferation through interaction with Rho but not Rac or Cdc42 (Eun et al. 2006). Both Plexin-D1 and Plexin-C1 function as R-Ras GAPs to inhibit migration, but differ in their requirement for Rnd2 to mediate this function (Uesugi et al. 2009). Both plexins serve similar functions yet are regulated in this function by Rnd2, allowing for very finely controlled regulation of R-Ras.

Obvious questions regarding the function of Plexin-B2 in regulation of small GTPases can be raised. Does Plexin-B2 regulate M-Ras and R-Ras similar to Plexin-B1? Does Plexin-B2 regulation of R-Ras require Rnd1 or Rnd2, and what are the downstream consequences? What differentiates Plexin-B2 signaling from Plexin-B1 signaling? Studies should examine other macrophage plexins and their influence on Rho family GTPases. It is possible that plexins interact with each other and/or with ligand to regulate RhoGTPase signaling? Studies have shown that Plexin-A1 and Plexin-B1 interact in their cytoplasmic domains, and are thought to cooperate in signaling (Usui et al. 2003). In zebrafish embryos the signaling pathways of Plexin-B2 and Plexin-D1 antagonize each other to control sprouting behavior of angioblast (Lamont et al. 2009). A complete understanding of how plexins as a group mediate cell movement, response to pathogen, proliferation, or survival cannot be ascertained by studying plexins individually. Studies of plexins as a family will provide a more complete understanding of the intricacies of plexin signaling.

Plexin-B2 and its Effects on Phagocytosis

In macrophages, FcRγ mediated phagocytosis requires Rac and Cdc42 (Cox et al. 1997). Data in Chapter 2 shows that *Plxnb2*$^{-/-}$ macrophages have more active Cdc42 and Rac in steady state than wild type. We hypothesized that Plexin-B2 may play a role in uptake of opsonized T cells, E. coli, or latex beads. However out data show that macrophages are not defective in their uptake of Ig-coated T cells, *E. coli*, or latex beads, suggesting that Plexin-B2 does not regulate signaling that occurs during Fc-mediated phagocytosis. These data demonstrate that Plexin-B1 control of Rac is very specific. It is possible that FcγR signaling, while dependant on Rac and Cdc42, functions independently of Plexin-B2 regulation of Rac signaling. Thus the two pathways may converge at Rac but not influence each other. These questions could be answered by examining how Plexin-B2 affects RacGTP levels during the phagocytic process. These results may show that Plexin-B2 regulation of Rac and Cdc42 functions independently of FcγR mediated modulation of Rac. Studies of Sema3A, which has been shown to inhibit actin cytoskeleton reorganization, does not affect phagocytosis in macrophages (Dent et al. 2004; Ji et al. 2009). However, Plexin-C1 has been shown to inhibit phagocytosis upon binding to its viral ligand Poxvirus A39R (Walzer et al. 2005). It is possible that because there is more already more active Cdc42 and Rac in *Plxnb2*$^{-/-}$ cells, and phagocytosis also induces activation of Rac and Cdc42, that additive active Rac and Cdc42 do not result in additional phagocytosis (Beemiller et al. 2010).

Plexin-B2 and Cytokine Secretion

The plexin family has been implicated in control cytokine secretion in the immune system. Plexin-A1 regulates Type I IFN-α through association with PDC-TREM in pDCs (Watarai et al. 2008). Sema4D deficient DCs are unable to produce IL-12 compared to wild

type DCs (Kumanogoh et al. 2002). Sema7A induces inflammatory cytokine production in macrophages through its integrin receptor $\alpha 1\beta 1$ (Suzuki et al. 2007). We hypothesized that Plexin-B2 may also regulate cytokine secretion in DCs and macrophages. Data in Chapter 2 shows that *Plxnb2*$^{-/-}$ macrophages secrete normal amounts of IL-6 and TNFα in response to TLR ligands Poly(I:C), LPS, and R837. Data presented in Chapter 3 shows that *Plxnb2*$^{-/-}$ ex-vivo dendritic cells secrete normal amounts of IL-6 and TNFα in response to LPS and anti-CD40 antibody, but are overactive in their response to IL-12/IL-23p40 both without stimulation and in response to LPS.

IL-12/IL-23p40 subunit contributes to the active forms of both IL-12 and IL-23, or can exist as a homodimer or monomer. The IL-12 pathways leads to a Th1 response, while the IL-23 pathway leads to induction of Th17 cells (reviewed in Gee et al. 2009). Intriguingly, the IL-12/IL-23p40 subunit has been shown to exist in-vivo as a monomer or dimer of itself and is present in excess of IL-12 or IL-23, and is suggested to function as a negative regulator if IL-23 and/or IL-12 signaling (reviewed in Cooper et al. 2007). The monomer form of IL-12p40 is required for dendritic cell migration in response to Mycobacterium tuberculosis infection (Khader et al. 2006). Determining the exact form of IL-12/IL-23p40 protein(s) that are negatively regulated by Plexin-B2 and Plexin-D1 is a critical next step in understanding this finding and its immune consequence.

These data in the context of data from the field demonstrate that plexin control of cytokine secretion is very specific. Plexins may couple environmental and attachment cues to inflammatory conditions so that cells respond only when in the correct location or in contact with the correct cells.

Plexin-B2 and Migration

Chapter 2 demonstrates the Plexin-B2 negatively regulates macrophage motility but not directed migration. Most plexins have been shown to mediate migration or motility. It is interesting that many cells express more than one plexin, yet most plexins mediate cell movement. Are different plexins activated at different locations within the body or within specific locations on the cell? Are plexins so important for cell movement that they are redundant? Do different cytokines or cell-cell contacts result in activation of different plexin signaling pathways? These questions are very complex and are not easily addressed by individual knockout studies, as simply taking out a gene may induce others or eliminate interactions of plexins with each other. Studies of Plexin-B2 and other plexins that are fluorescently labeled and can be observed in real time within the cell, similar to the biosensor studies used in the study of RhoGTPases at the leading edge of a cell (Machacek et al. 2009), would provide more answers to these complex questions.

Plexin-B2 and Cancer Biology

Mutations in Plexin-B1 that interfere with its ability to bind and regulate Rac have been identified in one study in 89% of percent of human prostate cancer samples with bone metastases (Wong et al. 2007). Plexin-B2 contains many of the same mutation sites as Plexin-B1, and may contribute to cancer by a similar mechanism. Plexin-B2 has been identified in the cells of vascular, brain, epithelial, and endocrine systems origin by immuno-staining of sections of tissue (Friedel et al. 2007; Zielonka et al. 2010) Thus mutations in Plexin-B2 could result in inappropriate regulation of many different cell types that have potential of overgrowth within the body.

Many other Plexins have been recently linked to cancer. Overexpression of Sema3E increases invasion and metastasis in tumor cells through its receptor Plexin-D1 in complex with ErbB2 (Casazza et al. 2010). Expression of Plexin-B3 and its receptor Sema5A increase from normal tissue to lymph node metastases in gastric carcinoma samples (Pan et al. 2009). Molecular profiling of melanoma and pancreatic cancer cells show that multiple plexins have loss of function mutations that increase progression (Casazza et al. 2010). These studies suggest that Plexins have regulatory roles across broad types of cancers. Understanding how the plexin interactions combine with other mutations in cancer may allow for development of drugs targeting the plexins or increased detection of metastatic cells. Regulating the activation state by use of their ligand or other biological mimic is a potential strategy for targeting cancer cells and their ability to move or invade.

Plexins and Semaphorins- Future Directions

One of the most critical questions remaining to be answered is why and if plexin proteins function differently depending on the cell type. Plexins can be tyrosine phosphorylated, are found at the cell surface, and can function in conjunction with growth factor receptors. This makes them potential drug targets for the treatment of diseases such as cancer and autoimmunity. However, it is critical to understand how the immune, nervous, and other systems mediate responses to plexins and semaphorins. For example, does Plexin-A1 function differently in the nervous system due to availability of ligand, expression of co-receptors, or expression differences of individual plexins or groups of plexins? B family plexins are capable of being cleaved by proprotein convertases in overexpression studies (Artigiani et al. 2003), suggesting that Plexin-B2 is capable of both long distance and local

signaling. Alternatively, secreted Plexin-B2 may function to block secreted semaphorin ligand(s), such as Sema4D. Understanding how plexin and semaphorin signaling is differentially regulated between different body systems may provide data that would allow for creation of drugs that target only plexin or semaphorin signaling in one body system but not others. For example, Semaphorin-3A has been tested as a drug treatment in a rodent model of atopic dermatitis, where it was found to reduce disease (Yamaguchi et al. 2008). The effects of Semaphorin-3A on the brain were not assessed in this study. Semaphorin-3A has been shown to induce cell death in cortical neurons of adult mice (Jiang et al. 2010), thus the side effects of Semaphorin-3A as a drug could be detrimental. However, if difference in signaling components between the two systems could be ascertained, a more targeted drug could be created. Future studies must address the roles of plexins and semaphorins across multiple body systems.

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