THE ROLE OF PLEXIN-D1 IN THE ADAPTIVE IMMUNE SYSTEM: IMPLICATIONS FOR HUMORAL IMMUNITY

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology, School of medicine

Chapel Hill

2010

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ABSTRACT

EDUARTA HOLL: The role of Plexin-D1 in the adaptive immune system: implications for humoral immunity
(Under the direction of Dr Jenny P.-Y Ting)

Plexin and semaphorin molecules direct axon localization by generating attractive and repulsive forces. Recently, plexin and semaphorin molecules have been shown to control cell movement and cell-cell interaction in the immune system. In this study, we characterize the expression and function of plexin-D1 in the immune system. We show that plexin-D1 is highly expressed in thymocyte, B cell and dendritic cell compartments. Absence of Plexin-D1 resulted in aberrant thymic development and impaired DP thymocyte migration from the cortex to the medulla. However, we found that absence of plexin-D1 did not affect the formation of mature T cell compartments and peripheral T cells were capable to respond to antigenic challenge. Normal B cell development and maturation was observed in *PlxnD1*$^{-/-}$ mice; however, these mice exhibited defective germinal center (GC) reactions during T-dependent immune activation. Protective humoral immune responses depend upon the generation of memory B cells and long-lived plasma cells formed in the GC reaction. We demonstrate that Plexin-D1 is responsible for migration of activated B cells into the GC. We correlate these observations with reduced migration of *PlxnD1*$^{-/-}$ B cells towards the GC chemokines, CXCL12, CXCL13 and CCL19. Accordingly, *PlxnD1*$^{-/-}$ mice exhibited defective production of IgG1 and IgG2b, but not IgG3 serum antibody, accompanied by reductions in high-affinity antibody, long-lived bone marrow plasmacytes, and recall

humoral memory response. These data show a new role for plexin molecules in the GC reaction and generation of immunologic memory.

ACKNOWLEDGMENTS

I sincerely thank my mentor, Dr. Jenny Ting for her continued support and guidance. I am thankful that she has allowed me to be part of her research group for the last five years. She provided me with all the resources I needed to complete my graduate work. My graduate school experience owes everything to the time and effort she has put into my training. Without her none of this would be possible.

I thank all of my committee members, Dr. Steve Clarke, Dr. Pat Flood, Dr. Jon Serody, and Dr. Lishan Su for all of their support, suggestions and constructive criticism throughout the years.

I thank all the Ting Lab members who have helped me these last five years. They have taught me how to perform experiments, analyze data and present my research. I thank them for all their time and effort.

Finally, I thank my husband Matt for all of his love, support and encouragement. His patience and understanding made this process possible. I love you!

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Ab	antibody
Ag	antigen
APC	antigen presenting cell
BMDC	bone-marrow derived mactorphages
CD	cluster of differentiation
CIITA	MHC class II transactivator
CCR	chemokine receptor
CCG	chicken gamma globulin
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
dsDNA	double stranded DNA
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanate
FO	follicular

FTOC	fetal thymic organ culture
GC	germinal center
HRP	horseradish peroxidase
i.p.	intraperitoneal
i.v.	Intravenous
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
KLH	keyhole limpet hemocyanin
MAb	monoclonal antibody
MHC	major histocompatibility complex
MZ	marginal zone
N-terminus	amino terminus
NP	4-hydroxy-3-nitrophenyl acetyl
OVA	ovalbumin
PBS	phosphate buffered saline
PFA	peraformaldehyde
PCR	polymerase chain reaction
PE	phyco-erythrin
PerCP	peridinin-chlorophyll protein
Plxn	plexin
RT-PCR	reverse transcription-polumerase chain reaction

SAV	streptavidin
SP	single positive
TCR	t cell receptor
Tg	transgenic
Th1	type 1 T helper
Th2	type 2 T helper
TNF	tumor necrosis factor
WT	wild type
α	alpha
β	beta
γ	gamma
κ	kappa
λ	lambda

CHAPTER I

INTRODUCTION

1.1 PLEXINS AND SEMAPHORINS OVERVIEW

Origins: Plexins and semaphorins were discovered over 20 years ago as molecules involved in cellular guidance. The nomenclature of semaphorins was derived from the analogous properties of these molecules to the system of flags and lights used in maritime communication (Kolodkin, Matthes et al. 1993; Luo, Raible et al. 1993; Tessier-Lavigne and Goodman 1996). Plexins were identified in Xenopus as molecules expressed in the plexiform layers of the neural retina (Takagi, Tsuji et al. 1987; Ohta, Takagi et al. 1992). Two groups independently later identified and named them plexin molecules (Ohta, Mizutani et al. 1995; Tamagnone, Artigiani et al. 1999).

Plexins and semaphorins are molecules required to direct axons to their specific targets via repulsive or attractive cues (Kolodkin, Matthes et al. 1993; Song, Ming et al. 1998; Winberg, Noordermeer et al. 1998). While these molecules were identified in the nervous system, their utilization in other biological systems has been established. Research on plexins and semaphorins has shown that these molecules are important in vasculature development, tumor biology, and immune regulation (Granziero, Circosta et al. 2003; Wong, Brickey et al. 2003; Gitler, Lu et al. 2004; Walzer, Galibert et al. 2005; O'Connor, Eun et al. 2008; Yamamoto, Suzuki et al. 2008). The impact of plexins and semaphorins in the immune system can elevate our understanding of immune cell-cell interaction, trafficking and activation. In this dissertation, I demonstrate and discuss the importance of plexins and semaphorins in the immune system, with particular focus in B cell biology.

Semaphorins: Semaphorins are large glycoproteins that function in secreted and membrane-bound form (Pasterkamp and Kolodkin 2003). All semaphorins contain the "sema domain", a conserved 500-amino acid extracellular domain (1999). The sema domain consists of a variant form of the seven-blade β-propeller fold, a highly conserved structure widely utilized for protein-protein interactions (Gherardi, Love et al. 2004). Over 20 semaphorins have been identified and are categorized into groups based on their origin and sequence homology: invertebrate, vertebrate and viral semaphorins (1999). The invertebrate semaphorin group is further divided into two classes, I and II; whereas, the vertebrate semaphorins are divided into groups III-VII (**Figure 1.1**). Class II, III, and VIII semaphorins are secreted whereas the rest are membrane bound (1999). Semaphorins range in size from 400-1000 a.a. depending on the presence of additional domains, such as immunoglobulin (class II, III, IV, and VII) and thrombosponin domains (class V) or GPI linkage sites (Mizui, Kumanogoh et al. 2009).

Semaphorins have two predominant classes of receptors; plexins and neuropilins (Takahashi, Fournier et al. 1999; Kruger, Aurandt et al. 2005). Neuropilins are transmembrane proteins (~900a.a.) that contain short intracellular domains and thus, lack intrinsic signaling capabilities (Takagi, Hirata et al. 1991). Instead, neuropilins form co-receptor complexes with other cell surface molecules to mediate signal transduction (Kruger, Aurandt et al. 2005). The majority of semaphorins signal through plexins alone, however, class III semaphorins, with the exception of semaphorin3E, require neuropilin co-receptors to properly function (Gu, Yoshida et al. 2005; Chauvet, Cohen et al. 2007). In addition to neuropilin and plexin receptors, semaphorins can signal through other receptors, such as CD72 and T-cell immunogobulin and mucin domain-containing protein 2 (TIM-2) (Shi,

Kumanogoh et al. 2000; Oinuma, Ishikawa et al. 2004). It is clear that semaphorins can transmit their signals through a series of receptors. Thus, it is expected that these multiple molecular interactions would lead to a variety of signal transductions, perhaps more complex than initially thought.

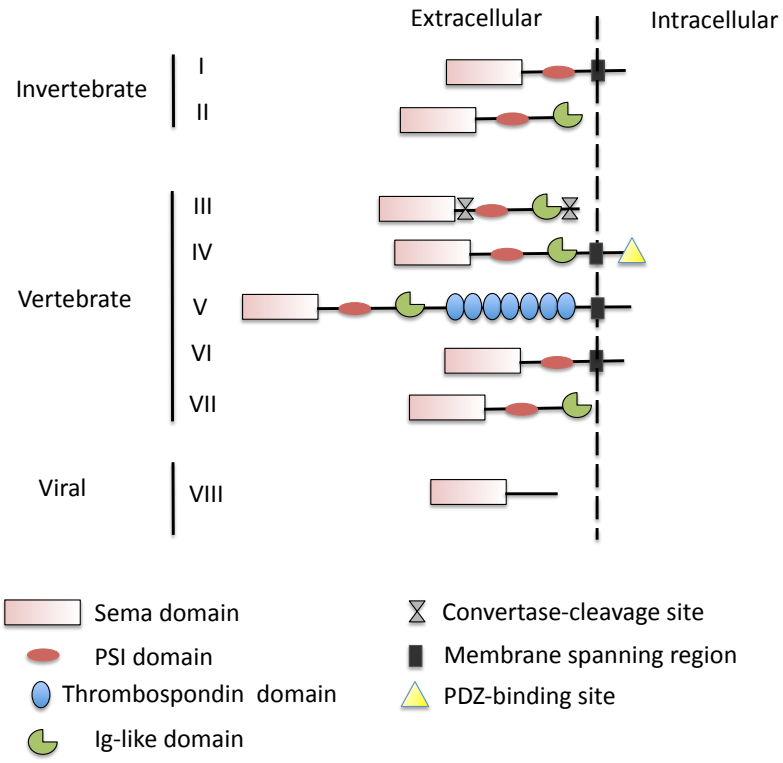

FIGURE 1.1 Semaphorins.
There are eight classes of semaphorins. Classes I and II are found in invertebrates, classes III-VII are found in vertebrates and class VIII semaphorins are encoded by viruses. All semaphorins are characterized by sema domains which are followed by plexin semaphorin integrin (PSI), thrombospondin, and Ig-like domains. They can be either secreted or membrane-bound.

Plexins: Plexins are a conserved family of large proteins (~200kDa) that are the canonical receptors for semaphorin molecules (Winberg, Noordermeer et al. 1998;

Takahashi, Fournier et al. 1999). Like semaphorins, plexins can be soluble or membrane bound (Tamagnone, Artigiani et al. 1999). Plexins are divided into four classes, A through D, based on their structural homology (Kruger, Aurandt et al. 2005). The invertebrate system contains two plexins, termed plexin-A and plexin-B. The vertebrate system contains four classes of plexins: class A (Plexin-A1, A2, A3, A4), class B (Plexin-B1, B2, B3), class C (C1), and class D (D1) (**Figure 1.2)**.

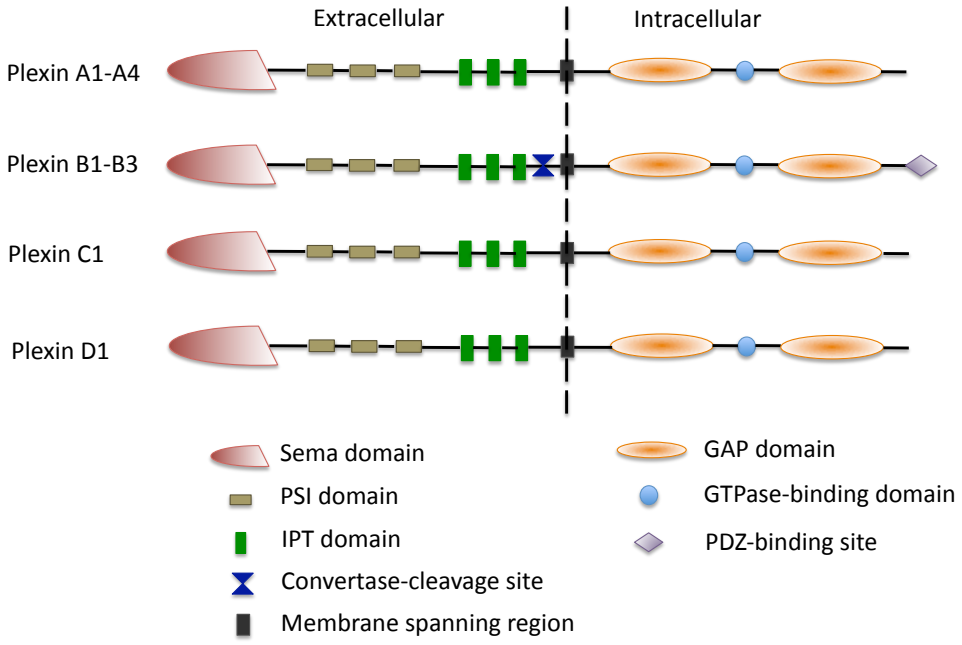

FIGURE 1.2 Plexins.
Plexins are divided into four different groups, A-D, based on their origin and structural homology. Class B plexins can be secreted whereas the rest of the plexins family members are membrane bound.

Plexins are structurally homologous to receptor tyrosine kinases, MET and RON, which play important roles in development, tissue regeneration and cancer (Gherardi, Love et al. 2004). The extracellular portion of plexin molecules contains a sema domain, followed by three plexin-semaphorin-integrin (PSI) domains and three Ig-like, plexins and transcription

factors (IPT) domains (Winberg, Noordermeer et al. 1998; Bork, Doerks et al. 1999; Antipenko, Himanen et al. 2003). Sema domains are responsible for ligand binding in plexin, semaphorin, and the proto-ongogenic MET and RON receptor tyrosine kinases (Antipenko, Himanen et al. 2003; Gherardi, Love et al. 2004). In addition to its ligand-binding capabilities, the sema domain acts as an autoinhibitory element to restrict activation of plexin molecules (Takahashi and Strittmatter 2001). For example, plexinA1 mutants that lack the sema domain are constitutively active and nonresponsive to ligand stimulation (Takahashi and Strittmatter 2001). Thus, the sema domains can be thought of as a regulatory element of plexin and semaphorin proteins that ensures proper activation.

The PSI domain has also been referred to as a small cystine-rich domain (CDR) or MET-related sequence (MRS) and is required for proper protein-protein interactions (Ohta, Mizutani et al. 1995). Xenopus studies of plexin-B2 were the first to suggest a role for the PSI domains of plexin molecules (Ohta, Mizutani et al. 1995). In these studies, it was suggested that ectopically expressed plexin proteins mediate cell adhesion via homophilic binding mechanisms of the PSI domains (Ohta, Mizutani et al. 1995). Conclusions on the role of PSI domains in plexin interactions were mainly based on previous studies of semaphorin3A (Klostermann, Lohrum et al. 1998). Cystine-rich repeats of semaphorin3A are responsible for disulfide bond formation in the semaphorin3A homodimer (Klostermann, Lohrum et al. 1998). To date, evidence for the role of PSI domains in plexin molecules has been sparse.

IPT domains, also referred to as Ig domains or transcription factor Ig domains (TIG) are necessary for ligand binding (Aravind and Koonin 1999). Studies blocking the IPT domain of the β2 integrin have suggested that these domains are required for dimerization of

the αβ subunits (Huang, Lu et al. 1997; Huang and Springer 1997). Other studies of MET proteins, lacking the sema domain, have revealed that the IPT domains alone are sufficient to ensure ligand binding (Basilico, Arnesano et al. 2008). Reports of RON proteins lacking an IPT domain region have demonstrated that absence of IPT domains causes hyperactive kinase activity of this protein and leads to colorectal cancer in humans (Wang, Kurtz et al. 2000; Zhou, He et al. 2003). Most of the research on plexin IPT domains has been done on plexin-A1. Studies of plexin-A1 have shown that absence of IPT domains inhibits association of this molecule with a triggering receptor on myeloid cells (TREM) family member and thus blocks downstream signaling (Watarai, Sekine et al. 2008).

The cytoplasmic portion of plexin molecules is essential for signal transduction upon ligand binding. Unlike, MET and RON tyrosine kinase receptors, the cytoplasmic tails of plexins do not exhibit kinase activity (Maestrini, Tamagnone et al. 1996). The intracellular domain of plexins is highly conserved among all classes and it shares homology with the intracellular domain of p120 RasGAP (Rohm, Rahim et al. 2000). Plexins contain a GTPase-binding domain and a segmented GTPase-activating protein (GAP) domain (Kruger, Aurandt et al. 2005). Many studies have focused on further elucidating the importance of plexin molecules in controlling GTPases (Rohm, Rahim et al. 2000; Oinuma, Ishikawa et al. 2004; Eun, O'Connor et al. 2006). These studies are of particular importance given the role of plexin molecules in cell movement, cytoskeleton rearrangement and synapse formation(Torres-Vazquez, Gitler et al. 2004; Eun, O'Connor et al. 2006).

In addition to all the components found in plexin molecules, plexin-B1, B2, and B3 also contain convertase-cleavage sites (Tamagnone, Artigiani et al. 1999). Cleavage and binding of these molecules to each other can result in formation of heterodimeric molecules

with enhanced activity (Artigiani, Barberis et al. 2003). Moreover, soluble plexin molecules can be released in the extracellular environment (Tamagnone, Artigiani et al. 1999), thus, eliminating the need for cellular proximity during plexin-semaphorin signaling events.

1.2 PLEXINS AND SEMAPHORINS IN THE IMMUNE SYSTEM

Plexin and semaphorin signaling has gained much interest in field of immunology. Immune responses consist of a series of spatiotemporally regulated events that implicate a number of different immune cell types. During these events, immune cells interact with each other to modulate their course of action. Studies of plexins and semaphorins have revealed that several members of these families are involved in a series of immune cell interactions, which ultimately influence the outcome of the immune response. Here we present the current knowledge of semaphorin and plexin molecules in the immune system.

Semaphorin4D and CD72 in the immune system: Semahorin4D is the first semaphorin molecule to be reported in the immune system and is highly expressed by T cells, mature DCs, and activated B cells (Delaire, Elhabazi et al. 1998; Kumanogoh, Watanabe et al. 2000; Shi, Kumanogoh et al. 2000; Kumanogoh, Suzuki et al. 2002; Granziero, Circosta et al. 2003; Kumanogoh, Shikina et al. 2005). Semaphorin4D has two known receptors, plexin-B1 and CD72, a C-type lectin (Huber, Kolodkin et al. 2003; Zhou, Gunput et al. 2008). The functional significance of semaphorin4D has been observed in both human and mouse immune systems. Initial studies of human semaphorin4D showed that this molecule promotes B-cell aggregation and differentiation (Hall, Boumsell et al. 1996). These studies were later

confirmed in the mouse where over-expression of semaphorin4D enhanced CD40- or LPS-induced proliferation and Ab production (Shi, Kumanogoh et al. 2000). Semaphorin4D regulates B cell immune responses by mitigating CD72 inhibitory signals and thus allowing downstream BCR signaling to proceed (Adachi, Flaswinkel et al. 1998; Kumanogoh, Watanabe et al. 2000; Kumanogoh, Shikina et al. 2005). The importance of semaphorin4D in B cell biology has been further demonstrated in studies utilizing semaphorin4D-deficient mice (Shi, Kumanogoh et al. 2000; Kumanogoh, Shikina et al. 2005). In these studies, B cell homeostasis is altered in the absence of semaphorin4D. These mice exhibit reduced B-1 B cell populations at a young age but increased numbers of marginal zone B cells later in life (Shi, Kumanogoh et al. 2000). Additionally, *Sema4D*$^{-/-}$ mice exhibit impaired T-dependent (Td) antibody responses and priming of antigen specific T cells.

Although T cells are the major semaphorin4D-producing cells in the immune system, semaphorin4D-deficient T cells develop normally and respond to antigenic stimuli (Shi, Kumanogoh et al. 2000). These studies suggest that semaphorin4D has no direct effect on T cells. Other *in vitro* studies have shown that semaphorin4D recombinant protein is important in inducing expression of CD80 and CD86 co-stimulatory molecules on DCs (Kumanogoh, Suzuki et al. 2002). Antigen specific *Sema4D*$^{-/-}$ T cells do not differentiate into effector cells when cultured in the presence of DCs. Together these data suggest that semaphorin4D expression on T cells is important to promote DC activation and maturation, which in turn promotes T cell activation (Kumanogoh, Suzuki et al. 2002). Thus, semaphorin4D exerts its function on T cells indirectly via DCs.

Disease models of autoimmunity in *Sema4D*$^{-/-}$ mice have revealed that this gene is important in controlling T-cell immune responses. Semaphorin4D deficient mice are resistant

to myelin oligodendrocyte glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE) (Ishida, Kumanogoh et al. 2003). A role for semaphorin4D in humans has been revealed outside the immune system. The absence of functional semaphorin4D can lead to death of immature neural cells in the spinal cord, likely, via its interaction with plexin-B1 (Giraudon, Vincent et al. 2004).

Semaphorin4A and TIM-2: Semaphorin4A is expressed on DCs and polarized T-helper type 1 (Th1) cells (Kumanogoh, Marukawa et al. 2002). The only identified receptor for semaphorin4A in the immune system is the T cell immunoglobulin and mucin domain-2 (TIM-2) receptor, a negative regulator of Th2 cells (Kumanogoh, Marukawa et al. 2002; Kuchroo, Umetsu et al. 2003; Chakravarti, Sabatos et al. 2005).

DC-derived semaphorin4A is required for adequate T-cell priming; however, semaphorin4A-deficient DCs develop normally and produce cytokines in response to various stimuli (Kumanogoh, Shikina et al. 2005). T-cell derived semaphorin4A is required for helper T cell differentiation (Th1) via T cell-T cell cognate interactions (Kumanogoh, Shikina et al. 2005). Indeed, mice deficient in semaphorin4A exhibit impaired Th1 responses to heat-killed *Propionibacterium acnes* (Kumanogoh, Shikina et al. 2005). However, these mice have enhanced responses to a Th2-inducing intestinal nematode, *Nippostrongylus brasiliensis* (Kumanogoh, Shikina et al. 2005). These studies suggest a requirement for semaphorinphorin4A in skewing of the T cell response towards a Th1 phenotype. *Sema4A*$^{-/-}$ mice show attenuated development of experimental autoimmune myocarditis, thus, further emphasizing the importance of this molecule in immune responses (Makino, Toyofuku et al.

2008). Additionally, *Sema4A*$^{-/-}$ mice on a BALB/c background are more prone to Th2 responses and develop spontaneous atopic dermatitis (Takamatsu, Okuno et al.).

Semaphorin6D and Plexin-A1: Semaphorin6D is expressed on T, B and NK cells (Takegahara, Takamatsu et al. 2006). Plexin-A1 is a well-studied receptor for semaphorin6D (Yoshida, Han et al. 2006) and was initially shown to be one of the gene products induced by CIITA (Wong, Brickey et al. 2003). Plexin-A1 is highly expressed by mature DCs but is low to undetectable in other immune cell types, such as macrophages, B cells and T cells (Wong, Brickey et al. 2003). Short hairpin RNA knock down studies demonstrated the functional significance of Plexin-A1 in DC biology (Wong, Brickey et al. 2003). DCs that lack expression of Plexin-A1 have a reduced capacity to activate T cells both *in vitro* and *in vivo*. These findings were confirmed in studies of *PlxnA1*$^{-/-}$ mice (Takegahara, Takamatsu et al. 2006). In addition, plexin-A1 associates with a molecular complex consisting of i) the triggering receptors expressed on myeloid cells-2 (TREM-2) and ii) the immuno-receptors tyrosine-based activation motif (ITAM)-bearing adaptor protein (DAP12). DCs deficient in TREM-2 or DAP12 behave similarly to plexnA1 deficient DCs (Takegahara, Takamatsu et al. 2006), indicating that these adaptor molecules are important mediators of semaphorin6D-induced plexin-A1 signaling.

Plexin molecules can impact the onset of autoimmunity diseases (Kumanogoh, Marukawa et al. 2002; Yamamoto, Suzuki et al. 2008). Plexin-A1-deficient mice are resistant to MOG-induced EAE (Takegahara, Takamatsu et al. 2006). In part, the generation of MOG-specific T cells is abrogated in plexin-A1-deficient DCs mitigating the induction of EAE upon exposure to MOG antigen. In addition, semaphorin6D has been implicated in the late

phase of T-cell responses (O'Connor, Eun et al. 2008) as CD4 T cells initiate expression of semaphorin6D by day4 post activation. Blocking semaphorin6D leads to decreased T-cell proliferation and inhibition of CD127 (IL-7 receptor) expression on T cells (O'Connor, Eun et al. 2008). Taken together these studies suggest that semaphorin6D and plexin-A1 partnering and signaling are likely to be implicated in regulation of late phases of T-cell immune responses and ultimately in generation of immunological memory.

Semaphorin7A, α1β1 integrin and plexin-C1: Semaphorin7A is a GPI-anchored protein expressed by $CD4^+CD8^+$ double positive thymocytes and activated T cells (Mine, Harada et al. 2000). Plexin-C1 and α1β1 integrin are two known receptors for semaphorin7A, (Pasterkamp, Peschon et al. 2003; Suzuki, Okuno et al. 2007). Activated T cells that express semaphorin7A can stimulate monocytes/macrophages through α1β1 integrin, inducing proinflammatory cytokine production (Suzuki, Okuno et al. 2007). *Sema7A*$^{-/-}$ mice are resistant to inflammatory diseases such as hapten-induced contact hypersensitivity and EAE (Suzuki, Okuno et al. 2007). In addition, semaphorin7A plays a critical role in TGF-β1-induced fibrosis, myofibroblast hyperplasia alveolar remodeling, and apoptosis (Kang, Lee et al. 2007). These studies indicate that semaphorin7A is important in regulating inflammatory immune responses.

Plexin-A4, Semaphorin3A, and Semaphorin6A: Plexin-A4 is expressed by T cells, DCs, and macrophages (Yamamoto, Suzuki et al. 2008) and interacts with at least two receptors, semaphorin3A and semaphorin6A (Suto, Tsuboi et al. 2007). *PlxnA4*$^{-/-}$ mice exhibit enhanced T-cell priming and exacerbated EAE disease (Yamamoto, Suzuki et al.

2008); however, the mechanisms by which plexin-A4 exerts its functions still remain unknown.

Plexin-B2: Plexin-B2 is not very well studied in the immune system. To date there has only been one report on the role of plexin-B2 in the immune system (Yu, Cook et al. 2008). Gene expression studies revealed that plexin-B2 is abundantly expressed on germinal center (GC) B cells. Plexin-B2 can be used to identify GCs, thus providing a new tool to study the GC reaction. The physiological relevance of plexin-B2 however, remains to be explored. Plexin-B2 may be required for recruitment and maintenance of activated B cells in the GC reaction.

Plexin-D1, Semaphorin3E and Semaphorin4A: Initially, plexin-D1 was identified for its key role in vasculature development (Gitler, Lu et al. 2004; Gu, Yoshida et al. 2005). *PlxnD1*$^{-/-}$ mice suffer congenital heart defects due to improper vessel patterning (Gitler, Lu et al. 2004; Gu, Yoshida et al. 2005). Studies of plexin-D1 in the immune system have been limited. Plexin-D1 is expressed by $CD4^{+}CD8^{+}$ "double positive" (DP) thymocytes and plexin-D1 has been shown to interact with semaphorin3E (Choi, Duke-Cohan et al. 2008) but not semaphorin4A (Toyofuku, Yabuki et al. 2007). *PlxnD1*$^{-/-}$ DP thymocytes are arrested in the cortex and seldom travel to the medulla (Choi, Duke-Cohan et al. 2008); however, the significance of plexin-D1 deficiency in the peripheral T cell immunity has not been determined. Since the elimination of autoreactive T cells occurs in the thymus, plexin-D1 deficiency may have a role in controlling the development of autoimmunity.

As described above, plexins and semaphorins are a new class of immunoregulatory molecules with distinct functions in various phases of the immune response. The effects of plexins and semaphorins are context- and cell differentiation state-dependent and many have complex influences in the immune system as a whole (**Table 1.1**). Several plexins and semaphorins are important in maintaining immunological homeostasis. Lack of these molecules can result in attenuated immune responses. Alternatively, several of these molecules are required to prevent autoimmune disorders. Despite this body of knowledge on the role of plexins and semaphorins in the immune system, more work remains to be done. This dissertation focuses on expanding our current knowledge on the importance of plexins and semaphorins on adaptive immune responses. We focus primarily on B- and T- cell development and activation with particular emphasis on the GC reaction.

Plexin/ Semaphorin	Expression	Binding Partner	Activities
Plexin-A1	DCs, plasmacytoid DCs	Semaphorin-6D	DC activation
Plexin-A4	T cells, DCs, Macrophages	Class 6 Semaphorins	Inhibition of T cell activation
Plexin-B2	GC B cells	Semaphorin-4A	Marks GCs
Plexin-D1	Double positive thymocytes	Semaphorin-4A and -3E	Thymocyte trafficking
Semaphorin-3A	T cells	Plexin-A family	Inhibits T-cell activation and monocyte migration
Semaphorin-4A	DCs, activated T cells, Th1 cells	Plexin-D1, Plexin-B2, Tim-2	T-cell activation and monocyte migration
Semaphorin-4D	T cells, activated B cells, DCs	CD72	B-cell activation and homeostasis, DC activation
Semaphorin-6D	T cells, B cells, NK cells	Plexin-A1	DC activation and production of type1 interferon, late-phase T cell proliferation
Semaphorin-7A	Activated T cells	Integrin $\alpha 1\beta 1$	Monocyte/macrophage activation

Table 1.1: Expression of plexin and semaphorin family members in the immune system

1.3 B CELL DEVELOPMENT

Immunoglobulin diversity: The bone marrow (BM) contains hematopoietic stem cells that give rise to lymphocyte and other blood cells populations (Thomas 1991). B-lymphocytes arise from committed lymphoid progenitors found in the BM. (Thomas 1991; Kondo, Weissman et al. 1997). These cells undergo a series of developmental stages in the BM and spleen to give rise to mature B cells (Pillai and Cariappa 2009).

The B-cell receptor (BCR) is necessary for B-lymphocyte survival and recognition of antigens during the course of an immune response (Carrasco and Batista 2006; Srinivasan, Sasaki et al. 2009)). The BCR is created by pairing of two different polypeptide chains; the immunoglobulin (Ig) heavy chain (HC) and light chain (LC) (Tonegawa 1983). During Ig HC rearrangement, gene segments of variable- (V), diversity- (D) and joining- (J) regions are assembled in a specific order (D-J, then V-DJ) (Tonegawa 1983). In-frame rearrangement of the HC and LC loci occurs when V(D)J rearrangement maintains the correct reading frame through the constant (C-) regions of each polypeptide (Schlissel 2004). After successful, in-frame completion of HC rearrangement, V- and J-gene segments are combined to form the LC. Together, the HC and LC form complementarity determining regions (CDRs) that bind antigenic structures in their native conformation (Carrasco and Batista 2006). (**Figure 1.3**)

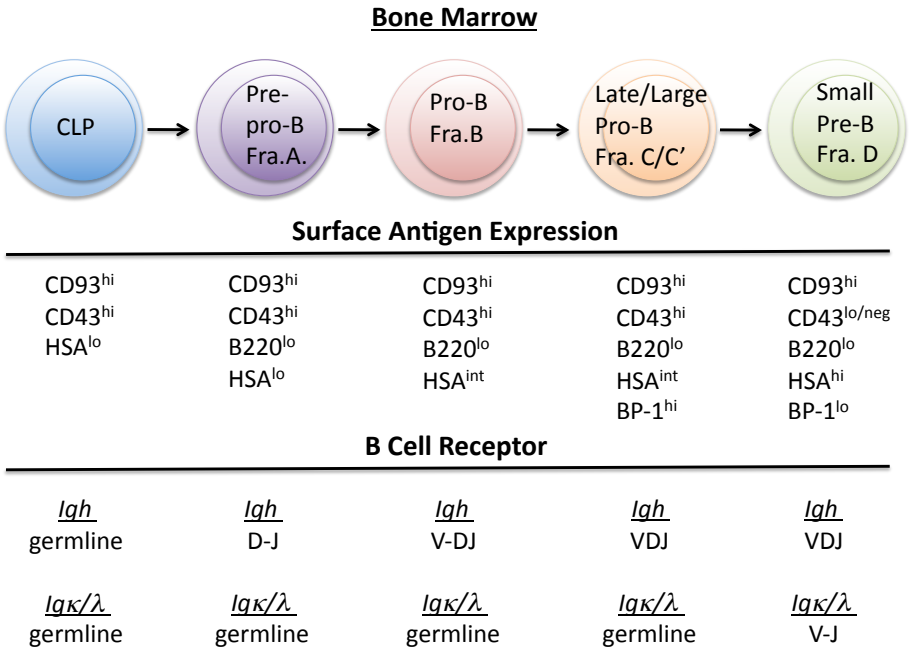

FIGURE 1.3 B-cell development in the bone marrow.
B cell precursors undergo a series of developmental processes where they rearrange V, D, and J gene segments of the HC and LC to give rise to immature B cells.

V(D)J recombination is crucial for the adaptive immune response to allow for a diverse array of antigen recognition. Dys-regulation of V(D)J recombination can lead to truncated non-productive Ig proteins as well as chromosomal translocations (Baumann, Potash et al. 1985). The B-cell repertoire comprises a diverse array of BCR molecules (~10^{14} distinct specificities) that promote survival of the host (Dickerson, Market et al. 2003). The usage of different C-regions (μ, δ, α, γ, and ε) determines the isotype of the antibody, each with unique biological properties (Stavnezer, Guikema et al. 2008). During class switch recombination, Cμ is replaced by downstream γ, α, or ε constant regions, yielding switched isotypes with the same antigen specificity and affinity (Stavnezer, Guikema et al. 2008). Cδ

expression occurs by alternative splicing of the Cμ-Cδ genes (Stavnezer, Guikema et al. 2008).

Pro- and pre-B-cell Development: Early stages of B-cell development are divided based on the rearrangement status of the Ig heavy (*Igh*) and light chain (*Igk or Igl*) loci (Chen and Alt 1993). During this developmental process, prepro-B cells rearrange and combine the D-gene segments to J-gene segments of *Igh* (Oltz 2001). Pro-B cells continue the rearrangement process by adding a V-gene segment to the already rearranged DJ segment (Oltz 2001). Pairing of the newly formed HC with surrogate LC leads to pre-BCR formation which is necessary for B-cell survival beyond the pro-B cell stage (Martensson, Rolink et al. 2002).

Furthermore, pre-B cells are divided into two populations, cycling large pre-B cells and non-cycling small pre-B cells (Martensson, Rolink et al. 2002). The large pre-B cell stage is characterized by increased proliferation of the cells with successful HC rearrangements. This in turn leads to increased numbers of small pre-B cells that can now rearrange their LC and further promote diversification of the BCR repertoire. Upon successful rearrangement of the LC, IgM antibody (HC+LC) is observed in the cytoplasm of small pre-B cells (Wang, Stephan et al. 2002) and in the cytoplasm and surface of immature B cells (Wardemann, Yurasov et al. 2003) while the IgD isotype is expressed later during development.

Additionally, early B-cell subsets are identified by flow cytometry using a classification system based on differential expression of surface antigens (Hardy, Carmack et al. 1991; Osmond, Rolink et al. 1998). In this system, VDJ rearrangement of the *Igh* loci is

determined in cells with the surface phenotype of prepro-B (fraction A-CD43^{hi}B220^{lo}HSA^{lo}BP-1^{neg}IgM^{neg}), early pro-B (fraction B-CD43^{hi}B220^{lo}HSA^{int}BP-1^{neg}IgM^{neg}), late pro-B and large pre-B (fraction C-C`-CD43^{hi}B220^{lo}HSA^{int}BP-1^{hi}IgM^{neg}) and small pre-B (fraction D-CD43$^{lo/neg}$B220^{lo}HSA^{hi}BP-1^{lo}IgM^{neg}) cells. This classification system is a valuable tool that allows for easy isolation of progenitor B cells for use in subsequent experiments.

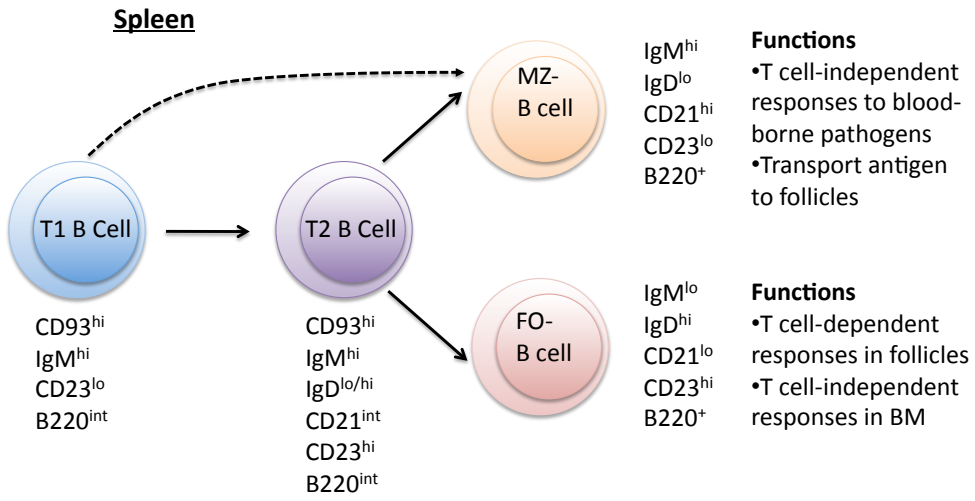

FIGURE 1.4 B-cell development in the spleen.
B cells go through T1 and T2 tranzitional stages which then give rise to MZ- and FO- B cells. Both cell types are used for different aspects of the humoral immune response.

Immature and transitional B-cell development: Immature and transitional B cell compartments are defined by the expression of surface molecules as well as distribution and localization in peripheral lymphoid organs (Pillai and Cariappa 2009). Immature and transitional B-cell populations are distinguished based on differential expression of fetal stem-cell antigen (CD93), IgM, IgD, complement- (CD21) and Fc-receptors (CD23) (Chung, Silverman et al. 2003; Casola 2007). Immature B cells are present in both the BM and spleen

and express a surface antigen profile ($B220^{int}CD21^{neg}CD23^{neg}CD93^{hi}IgM^{lo}IgD^{neg}$) that is low for BCR and other receptors (CD21 & CD23) whose cross-linking leads to mature B cell activation (King and Monroe 2000). BCR cross-linking in immature B cells leads to negative selection as these cells undergo apoptosis upon cognate antigen exposure (King and Monroe 2000). Immature B cells migrate from the BM to the spleen where they differentiate through transitional (T) stages into mature B cells capable of responding to antigen (Loder, Mutschler et al. 1999; Petro, Gerstein et al. 2002). Transitional type 1 (T1) B cells are present in BM and spleen and they give rise to transitional type 2 (T2) B cells which are found only in the primary follicles of the spleen (Chung, Sater et al. 2002). T1 B cells ($B220^{int}CD21^{neg}CD23^{lo/neg}CD93^{hi}IgM^{hi}IgD^{neg}$) do not express IgD BCR, CD21 or CD23 antigens that are present on T2 B cells ($B220^{int}CD21^{int}CD23^{hi}CD93^{int}IgM^{hi}IgD^{lo/hi}$). Unlike T1 B cells, T2 B cells are characterized as responsive to BCR engagement which results in increased proliferation and expression of the prosurvival protein Bcl-x(L).

Mature B-cell compartments: Mature B-cell compartments include three distinct populations; mature follicular (FO), marginal zone (MZ), and B1 B cells. FO B cells are located in follicles adjacent to T-cell zones; this localization promotes cell-cell interaction and migration towards each other when activated to participate in T-dependent immune responses (Han, Zheng et al. 1995). FO B cells express high levels of IgD and CD23, but low levels of IgM and CD21 ($B220^{+}CD23^{hi}CD21^{lo}IgM^{lo}IgD^{hi}$). They circulate and are found in the BM, blood, lymph nodes (LN) and mucosal areas where they encounter foreign antigen (Pillai and Cariappa 2009). (**Figure 1.4**)

MZ B cells do not circulate in the bloodstream of mice and are long-lived cells that do not need constant replenishment from progenitor cells of the BM (Hao and Rajewsky 2001). They are located between the white pulp (B- and T-cell zones) and the red pulp (Dono, Zupo et al. 2003; Lopes-Carvalho and Kearney 2004). MZ B cells express high levels of IgM and CD21, but low levels of IgD and CD23 ($B220^{+}IgM^{hi}IgD^{lo}CD23^{lo}CD21^{hi}$) (Lopes-Carvalho and Kearney 2004; Pillai, Cariappa et al. 2005). In addition to B cells, the MZ includes metallophilic macrophages and dendritic cells (DC), surrounding the follicular mantle (Martin and Kearney 2002). MZ B cells respond to antigenic signals more rapidly than FO B cells and are involved in the initial control of pathogen expansion (Snapper, Yamada et al. 1993; Oliver, Martin et al. 1997).

B1 B cells are primarily found in pleural and peritoneal cavities with very low frequencies observed in circulation and secondary lymphoid tissues (Hayakawa, Hardy et al. 1985; Lalor, Stall et al. 1989). The origin of self-renewing B1 B cells remains controversial as several reports suggest that these cells arise strictly from fetal liver precursors, whereas other reports have suggested that splenic B cells of the B2 phenotype can differentiate into B1 B cells (Hardy, Carmack et al. 1991; Bhat, Kantor et al. 1992; Qian, Santiago et al. 2001). B1 B cells are distinguished from other B cell populations based on a distinctive pattern of cell surface marker expression ($B220^{lo}IgM^{hi}IgD^{lo}CD23^{lo}CD21^{lo}CD11b^{lo}$). B1 B cells are further subdivided into B1a ($CD5^{+}$) and B1b ($CD5^{-}$) subsets(Stall, Adams et al. 1992). B1 B-cell populations are a major source of circulating natural antibody present in the serum and are thought to form a link between innate and adaptive immunity (Nakamura, Burastero et al. 1988; Nakamura, Burastero et al. 1988; Martin and Kearney 2001). As a front line of

immune defense, B1 B cells respond quickly to antigen exposure and promote host survival by controlling initial pathogen burden (Ochsenbein, Fehr et al. 1999).

1.4 B-CELL ACTIVATION

T-independent immune responses: B cell immune responses can be divided into two categories, those that require helper T cells (T_h), termed T-dependent (T_d) B-cell immune responses and those that can occur in the absence T_h cells, termed T-independent (T_i) B-cell immune responses (Fagarasan and Honjo 2000). T_i antigens are divided into T_i Type-1 (T1) and T_i Type-2 (T2) antigens based on their different mechanisms for B-cell activation. Most T1 antigens are polyclonal B cell activators, which include TLR ligands such as LPS, while T2 antigens are repeating epitopes of carbohydrate moieties found in pathogenic bacteria such as *Haemophilius influenzae* b**,** *Streptococcus pneumoniae and Neissera meningitides.* T2 antigens activate B cells by cross-linking of the BCR, resulting in short-lived plasma cells (Mond, Lees et al. 1995; Mond, Vos et al. 1995).

MZ B cells are early responders that differentiate into AFC upon interaction with blood-borne bacterial antigens. MZ B cells recognize and respond to antigen challenge within hours by proliferating and forming plasmablasts (Lopes-Carvalho and Kearney 2004). More recently, it has been demonstrated that MZ B cells engage T_d immune responses by migrating to the T-B interface of the spleen and act as antigen presenting cells (APCs) (Attanavanich and Kearney 2004). It is widely agreed that B-cell memory is the result of T_d B-cell responses (McHeyzer-Williams and McHeyzer-Williams 2005); however, recent work suggests that some T_i type-2 antigen immunizations can form antigen-specific memory B

cells (Obukhanych and Nussenzweig 2006). These studies suggest that the T_d and T_i immune responses are not as distinct as previously described and the importance of MZ B cells extends beyond the first few hours of days of the immune response.

T-dependent immune responses: During the course of a T_d immune response, activated B cells migrate to nearby T-cell zones to interact with activated T_h cells. Upon encounter with T cells, the activated B cells receive survival, proliferation and differentiation signals (Dal Porto, Haberman et al. 1998; Garside, Ingulli et al. 1998; Dal Porto, Haberman et al. 2002).

The initial T_d response produces low affinity antigen-specific IgM or IgG from short lived plasma cells that reside in the spleen (Jacob, Kelsoe et al. 1991; Liu, Zhang et al. 1991; Allen, Okada et al. 2007). The GC reaction, which develops more slowly, gives rise to memory B cells and long lived BM plasma cells, the source of rapid and robust serum antibody responses characteristic of memory responses (Jacob, Przylepa et al. 1993; Allen, Okada et al. 2007). Thus, humoral immune responses are dependent on the quality, frequency, and location of antigen-specific, naive B cells.

1.5 THE GERMINAL CENTER REACTION

Overview: The GC reaction is the hallmark of T_d humoral immune responses and this GC reaction is pivotal in production of high affinity antibody and memory B-cell compartments (Kelsoe 1996; Wolniak, Noelle et al. 2006). The GC is comprised of B cells, T

cells, follicular dendritic cells (FDCs) and macrophages (Liu, Zhang et al. 1991; MacLennan 1994; Kelsoe 1996; Camacho, Kosco-Vilbois et al. 1998).

Previous studies suggest that activated B cells, specific for a foreign antigen, seed the GC within a few days after the initiation of the immune response (Jacob, Kassir et al. 1991; Liu, Zhang et al. 1991; Wang and Carter 2005). These cells initiate a complex cellular program that promotes proliferation, somatic hypermutation (SHM), isotype switching and differentiation into AFC and memory cells (McHeyzer-Williams, Driver et al. 2001). Activated B cells require additional stimulation (CD40-CD40L) from T_h cells in order to enter the GC reaction. Disruption of ligation of CD40 by CD40L blocks the formation of GCs (Han, Zheng et al. 1995). Indeed, genetic mutations in the *CD154* gene (CD40L) lead to elevated serum IgM, lack of class-switched antibodies and reductions in affinity maturation and B-cell memory (Aruffo, Farrington et al. 1993).

During the course of the GC response, FDCs capture antigen in the form of immune complexes, via CD21, and present this antigen to B cells. Additionally, FDCs secrete cytokines that further support B-cell activation and proliferation (Burton, Conrad et al. 1993; Wu, Qin et al. 1996; Tew, Wu et al. 1997). Activated FDC networks in the GC express vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which bind very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) respectively, on GC B cells (Koopman, Keehnen et al. 1994; Balogh, Aydar et al. 2002). These interactions are required to prevent B-cell apoptosis and support B-cell differentiation (Koopman, Keehnen et al. 1997).

The GC is divided into two distinct compartments; the light and dark zone (Close, Pringle et al. 1990). The dark zone is characterized by rapidly proliferating B cells

(centroblasts), which undergo SHM. The light zone is populated by FDCs that present antigen to activated B cells and facilitate clonal selection (Kelsoe 1996). Initial studies have suggested rapid movement of GC B cells between the light and dark zones in order to facilitate proliferation, mutation and clonal selection (MacLennan 1994; MacLennan 1994; MacLennan, Casamayor-Palleja et al. 1997; Allen, Ansel et al. 2004). However, recent studies have shown that clonal selection is not restricted to the light zone and can effectively occur in the dark zone (Hauser, Junt et al. 2007). These studies also demonstrate that GC B cells located in the dark zone are morphologically similar to B cells found in the LZ (Allen, Okada et al. 2007; Hauser, Junt et al. 2007). Moreover, these studies indicate that the predominant movement of GC B cells occurs as intrazonal circulation; while earlier studies suggested DZ to LZ interzonal trafficking. Circulating B cells are frequently found in the periphery of the GC where they receive T-cell help (Allen, Okada et al. 2007; Hauser, Junt et al. 2007). Together these data suggest that our understanding of the GC reaction is incomplete and future studies are needed to elucidate the mechanisms that control the GC formation and maintenance.

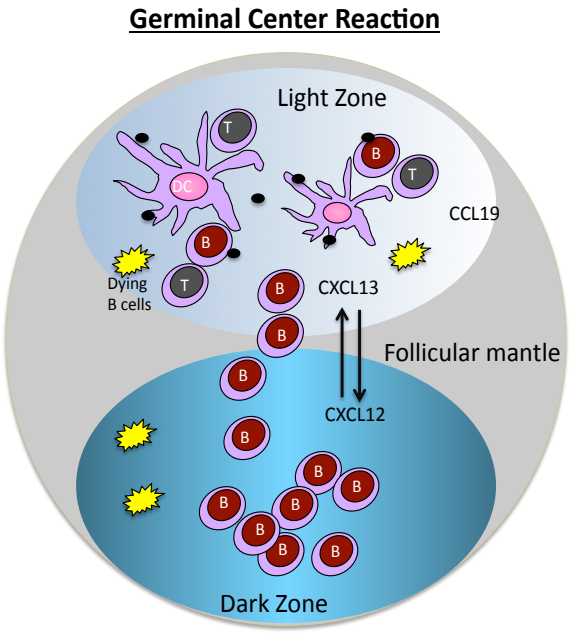

FIGURE 1.5. The GC reaction.
The GC is divided into LZ and DZ and different chemokines are needed for movement of B cells in between and within these zones.

Chemokines as key regulators of the GC reaction: The chemokine receptors such as CXCR4 and CXCR5 control formation and maintenance of GC (Freedman, Munro et al. 1990; Forster, Mattis et al. 1996; Arnold, Campbell et al. 2007). CXCL13 is expressed by FDCs and directs CXCR5-expressing B cells to the light zone (Allen, Ansel et al. 2004). CXCL12 is produced at higher concentrations by splenic stroma in the dark zone and has been suggested to control the recruitment of CXCR4-expressing B cells into the dark zone (Allen, Ansel et al. 2004). (**Figure 1.5**)

Productive GC responses generate high affinity antigen-specific B cells and long-lived memory B cells, resulting in enhanced protection upon secondary pathogen challenge. B cells that express high affinity B cell receptors have undergone somatic hyper mutation (SHM) (Jacob, Kassir et al. 1991; Jacob, Kelsoe et al. 1991; Muramatsu, Kinoshita et al. 2000). SHM requires activation-induced cytidine deaminase (AID); a B cell-specific enzyme

that is produced upon B-cell activation (Muramatsu, Kinoshita et al. 2000; Bransteitter, Sneeden et al. 2006). SHM yields mutations in the variable region of the Ig HC and LC. This process gives rise to antibodies with varying specificities and affinities for antigen (Manser, Wysocki et al. 1987; Jacob, Miller et al. 1992). High affinity B cell clones receive survival and differentiation signals from T_h cells and FDCs, whereas low affinity or self-reactive clones are purged (Berek, Berger et al. 1991; Han, Zheng et al. 1995). The process of selecting and expanding high affinity clones is termed affinity maturation (Berek, Berger et al. 1991; Han, Zheng et al. 1995; Shokat and Goodnow 1995).

1.6 CLASS SWITCH RECOMBINATION

CSR is an AID-mediated DNA recombination event, which diversifies antibody effector functions by replacing the Cμ gene segment (IgM antibody) of *Igh* with a targeted downstream constant region, such as Cα, Cγ, or Cε gene segments (IgA, IgG or IgE antibodies) (Stavnezer 2000; Manis, Tian et al. 2002). CSR occurs in the GC and requires CD40-CD40L interaction on B and T cells (Renshaw, Fanslow et al. 1994). IgA, -G, and -E Ab production is severly reduced in individuals containing a mutated CD40L gene, resulting in hyper-IgM syndrome. Recently, CSR has been shown to occur in *CD40*$^{-/-}$ mice, in T-independent splenic MZ and intestinal lamina propria immune responses (Renshaw, Fanslow et al. 1994; Fagarasan and Honjo 2000). These findings suggest that molecules other than CD40 can initiate CSR. Recent work has implicated the tumor necrosis factor (TNF) family member, BAFF, in CD40-independent CSR (He, Xu et al. 2007). (**Figure 1.6**)

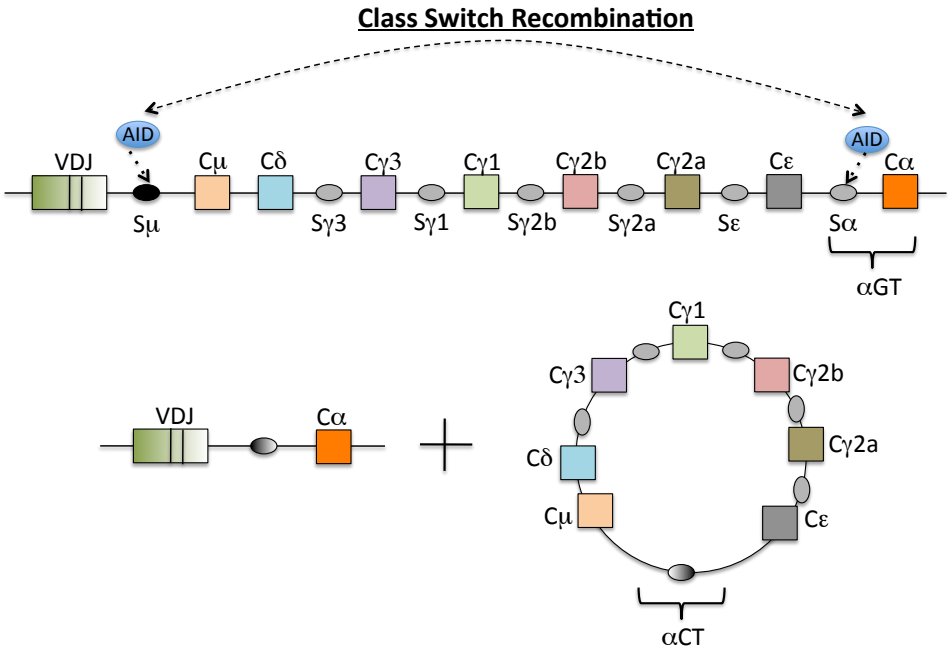

FIGURE 1.6. Class switch recombination.

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1.7 T-CELL DEVELOPMENT

T cells develop in the thymus from blood-borne hematopoietic precursors (Robey and Schlissel 2003). CD4 and CD8 double negative (DN) cells represent the earliest committed T cell precursor (Singer and Bosselut 2004). These cells can be further divided into four different classes (DN1-DN4) based on their expression of CD44 and CD25 (Penit, Lucas et al. 1995). DN thymocytes proceed through a series of maturation stages to give rise to double positive (DP) cells, which then serve as the precursor pool for CD4 and CD8 T cells (Penit, Lucas et al. 1995). Thymic localization is required to ensure proper T cell development and education (Benoist and Mathis 1999). DN thymocytes are found in the subcapsular (outer cortical) region whereas, DP cells are predominantly found in the inner cortical region where

they undergo “positive” selection for appropriate MHC restriction. The thymus contains a large number of specialized epithelial cells that can act as antigen presenting cells (Rossi, Jenkinson et al. 2006). Thymocytes that survive positive selection migrate to the medulla to complete thymic maturation. Autoreactive cells are eliminated in the medulla by negative selection (Shortman, Vremec et al. 1998). Additionally, the medulla is populated by antigen-presenting dendritic cells, which aid in the process of negative selection (Anderson and Jenkinson 2001).

Chemokines are key regulators of thymocyte immigration, emmigration and between the thymic compartments (Ansel and Cyster 2001). For example, CXCL12 is a key chemokine expressed by stromal cells in the thymic cortex (Suzuki, Sawa et al. 1999) to recruit early thymic progenitors that express CXCR4 and ensure cortical localization (Plotkin, Prockop et al. 2003). $CD25^{int}CD44^{+}$ DN thymocytes express CCR7 and migrate to the outer thymic cortex in response to CCL19 and CCL21 chemokines (Misslitz, Pabst et al. 2004). Upon TCR-mediated positive selection, single positive thymocytes elevate expression of CCR7 and CCR9 and migrate from the cortex into the medulla (Ueno, Saito et al. 2004), a process that allows continued thymocyte differentiation and selection. These studies suggest that thymocyte trafficking and T-cell selection are complex processes that are not very well understood. Although many of the chemokines studies have proven to be important in cell movement, they do not account for all the thymocyte movement patterns that are observed during T cell selection. Future work should focus on identifying novel molecules that control thymocyte migration and T cell selection.

1.8 DC DEVELOPMENT AND FUNCTION

DCs are highly efficient antigen presenting cells (Cella, Sallusto et al. 1997). DCs are important in mediating positive and negative selection of T cells in the thymic medulla (Hanahan 1998) as well as in eliciting successful immune responses against microbial pathogens in peripheral lymphoid organs (Cella, Sallusto et al. 1997). To date, three types of DCs have been identified based on their cell surface marker expression: lymphoid DCs (CD11c+CD11bLowCD8+, CD205Hi, Mac-1Low), myeloid DCs (CD11c+CD11b+CD8-CD205-), and plasmacytoid DCs (mPDCA-1+, B200+, Ly6C) (Liu and Nussenzweig ; Liu, Waskow et al. 2007; Bogunovic, Ginhoux et al. 2009; Ginhoux, Liu et al. 2009). The different subtypes of DCs have specific anatomical locations, thus, giving them distinctive functions (Liu, Waskow et al. 2007). For example, lymphoid DCs are found in the thymic medulla and the splenic T cell zone, where they induce T cell tolerance and prevent T cell responses to self-antigen (Shortman, Vremec et al. 1998). Myeloid DCs are found in the marginal zone of the spleen where they readily take up blood-borne foreign antigen (Balazs, Martin et al. 2002). They process the antigen and transport it to the rest of the spleen to promote T-cell activation or B cell GC responses (Balazs, Martin et al. 2002). pDCs are often referred to as IFN-producing cells due to their ability to produce high levels of type I IFN upon viral challenge or during the course of autoimmune disease (Martin, Del Hoyo et al. 2002; Asselin-Paturel, Brizard et al. 2005; Gilliet, Cao et al. 2008).

1. 9 CONCLUDING REMARKS

Recent work has enhanced our understanding of the role of plexin and semaphorin molecules in cell-cell communication in the immune system. The aim of this dissertation was to better understand the role of plexin-D1 in B cell, T cell, and DC development and activation during the course of the immune response. In chapter 2, we investigated the role of plexin-D1 in humoral immunity. We found that plexin-D1 is important for establishment and maintenance of a successful GC reaction. Plexin-D1 contributes to GC formation by facilitating recruitment of activated B cells into the GC reaction in response to CXCL12, CXCL13 and CCL19 chemokines. Next, we focused on antibody responses in the absence of plexin-D1. We found that short-term antibody responses were unaffected in the absence of plexin-D1; however, recall humoral immune responses were impaired. The aim of chapter 3 was to investigate the role of plexin-D1 in T cell development and activation. We found that plexin-D1 was important for thymocyte migration from the cortex to the medulla. Plexin-D1 was dispensable for peripheral T cell and DC maturation and function. This body of work adds to our current knowledge of immune plexins and semaphorins. We find plexin-D1 to be required for efficient cell movement and localization during humoral immune responses. This finding contribute to our current understanding of the GC and could have potential implications for future vaccine design aimed at modulation of the GC response and the memory B cell compartment.

CHAPTER II

PLEXIN-D1 REGULATES HUMORAL IMMUNE MEMORY VIA CONTROL OF B-CELL ENTRY INTO THE GERMINAL CENTER

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2.1 ABSTRACT

Long-lived humoral immune responses depend upon the generation of memory B cells and long-lived plasma cells during the germinal center (GC) reaction. These memory compartments, characterized by class-switched IgG and high-affinity antibodies, are the basis for successful vaccination. We report that a new member of the plexin family of molecules, Plexin-D1, controls the GC reaction and is required for secondary humoral immune responses. Plexin-D1 was not required for B cell maturation, marginal-zone precursor development, dark and light zone formation, $Ig\lambda^+$ and $Ig\kappa^+$ B cell skewing, B1/B2 development and the initial extra-follicular response. Plexin-D1 expression was increased following B cell activation and *PlxnD1*$^{-/-}$ mice exhibited defective GC reactions during T-dependent immune activation. *In vivo, PlxnD1*$^{-/-}$ B cells accumulated around the GC, but failed to efficiently enter the GC. *PlxnD1*$^{-/-}$ B cells showed a defect in migration towards the GC chemokines, CXCL12, CXCL13 and CCL19. Accordingly, *PlxnD1*$^{-/-}$ mice exhibited defective production of IgG1 and IgG2b, but not IgG3 serum antibody, accompanied by reductions in high-affinity antibody, long-lived bone marrow plasmacytes, and recall humoral memory response. These data show a new role for immune plexins in the GC reaction and generation of immunologic memory.

2.2 INTRODUCTION

Plexins were initially identified as molecules involved in axon guidance during neuronal development (Kolodkin, Matthes et al. 1993; Winberg, Noordermeer et al. 1998). Emerging research on plexins has revealed that many of these family members are indispensable in developmental programs of the immune system (Suzuki, Kumanogoh et al. 2008) and are required for proper immune activation (Granziero, Circosta et al. 2003; Wong, Brickey et al. 2003; Walzer, Galibert et al. 2005; O'Connor, Eun et al. 2008; Yamamoto, Suzuki et al. 2008). Plexin-D1 was first shown to be important for its role in vasculature formation as well as heart development (Gitler, Lu et al. 2004; Gu, Yoshida et al. 2005), but recent work demonstrates its role in controlling migration of double positive (DP) thymocytes (Choi, Duke-Cohan et al. 2008). Plexins have different ligands by which they exert their function. The primary ligands for the plexin family of molecules are the semaphorins (Suzuki, Kumanogoh et al. 2008). Plexin-D1 has two known ligands, semaphorin-4A and semaphorin-3E (Chauvet, Cohen et al. 2007; Choi, Duke-Cohan et al. 2008). However, semaphorin-3E, is the only plexin-D1 ligand that has been identified in immune tissues (Choi, Duke-Cohan et al. 2008).

We show that plexin-D1 is most highly expressed by activated B cells and has significant effects on their migration, capacity to form germinal centers (GCs) and secondary antibody responses.

Successful activation of B cells during a T-dependent (T_d) immune response is essential in providing long-term immunity (Jacob, Kassir et al. 1991; Blink, Light et al. 2005). The T_d response results in follicular (FO) B-cell activation and subsequent plasma cell development and GC formation (Jacob and Kelsoe 1992). The initial T_d response results in

low affinity antigen-specific IgM or IgG production by short lived plasma cells that reside in the spleen (Jacob, Kelsoe et al. 1991; Liu, Zhang et al. 1991; Allen, Okada et al. 2007). The GC reaction, which develops more slowly, gives rise to memory B cells and long lived bone marrow plasma cells which provide the rapid and robust serum antibody responses characteristic of secondary, memory responses (Jacob, Przylepa et al. 1993; Allen, Okada et al. 2007). GCs are the primary sites for affinity maturation, which is characterized by clonal expansion and somatic hypermutation (Jacob, Przylepa et al. 1993).

Whereas many studies have addressed the GC response and the types of mechanisms involved in its initiation and maintenance, we lack a complete understanding of this process. Earlier work suggested that the GC is divided into two distinct compartments; the light and dark zone (Close, Pringle et al. 1990). The dark zone is characterized by rapidly proliferating B cells (centroblasts), which additionally undergo somatic hypermutation. The light zone is populated by follicular dendritic cells which present antigen to activated B cells and facilitate clonal selection (Kelsoe 1996). This view has recently been revised as new data suggest that clonal selection can also occur in the dark zone (Hauser, Junt et al. 2007). GC B cells found in the DZ and LZ are not as morphologically different as originally thought (Allen, Okada et al. 2007; Hauser, Junt et al. 2007). Additionally, these studies report intrazonal circulation of GC B cells and substantial traffic along the GC periphery where B cells are thought to travel and receive T cells help (Allen, Okada et al. 2007; Hauser, Junt et al. 2007).

Previous studies have suggested that activated B cells specific for a foreign antigen seed the GC within a few days after the initiation of the immune response (Jacob, Kassir et al. 1991; Liu, Zhang et al. 1991; Wang and Carter 2005). B cells are then selected and continue on as long-lived plasma cells or memory B cells (Allen, Ansel et al. 2004; Hauser,

Junt et al. 2007; Schwickert, Lindquist et al. 2007). Chemokines are key regulators of the GC reaction: The chemokine receptors such as CXCR4 and CXCR5 control formation and maintenance of GC (Freedman, Munro et al. 1990; Forster, Mattis et al. 1996; Arnold, Campbell et al. 2007); CXCL13 and its receptor CXCR5 direct activated B cells to the GC light zone and are required for proper positioning of the GC light zone (Allen, Ansel et al. 2004), and; CXCL12 is thought to be required for the recruitment of CXCR4-expressing GC B cells from the light zone into the dark zone (Allen, Ansel et al. 2004). Although the identification of key chemokines and their receptors have furthered our understanding of the GC reaction, we still lack a complete picture of the molecular mechanisms involved in guiding B cells into and out of the GC reaction. Indeed, gene deletion studies focusing on CXCR4 and CXCR5-dependent mechanisms have suggested that GC formation is influenced by other, yet-to-be identified guidance molecules which traffic activated B cells in and out of the GC (Allen, Ansel et al. 2004; Arnold, Campbell et al. 2007; Hauser, Junt et al. 2007; Schwickert, Lindquist et al. 2007).

A gene profiling report found plexinB2 as one of the genes that are expressed by GC B cells although the physiological relevance of this finding was not explored (Yu, Cook et al. 2008). Plexins and their receptors, semaphorins, play significant roles in cell migration and positioning in other immune organs and are, therefore, likely candidates to play important roles in formation and maintenance of the GC (Suzuki, Kumanogoh et al. 2008). Thus, it is important to explore the physiological and functional role of plexins in the GC reaction.

This report reveals that plexin-D1 is expressed by B lymphocytes and that expression is greatly enhanced in activated B cells. Moreover, *PlxnD1*$^{-/-}$ B cells fail to migrate efficiently into GCs *in vivo*. Additionally, recall immune responses are attenuated in the

absence of plexin-D1 leading to defective antibody production. These results indicate a novel physiologic role of plexin-D1 in B cell activation.

2.3 RESULTS

PlxnD1 is predominantly expressed in B cells and upregulated by B-cell activation.

Expression of plexin-D1 in the immune system has not been fully characterized. *PlxnD1* was recently shown to be expressed by double positive (DP) thymocytes and controlled movement within the thymus (Choi, Duke-Cohan et al. 2008), indicating a role for *PlxnD1* in lymphocyte development and migration. We extended plexin-D1 expression studies from immature thymocytes to peripheral T and B cells using RT-PCR (**Figure 2.8A**) and quantitative real-time PCR (**Figure 2.8B**). *PlxnD1* mRNA expression is minimally detected in peripheral CD4 and CD8 T cells and in mature T cells that have been cultured under Th1 and Th2 skewing conditions. To characterize plexin-D1 expression in B cells, splenic (FO) ($B220^{+}IgD^{hi}IgM^{lo}CD21^{lo}CD23^{hi}$) and marginal zone (MZ) ($B220^{+}IgD^{lo}IgM^{hi}CD21^{hi}CD23^{lo}$) B cells were isolated by flow cytometry. As shown by both RT-PCR and real-time PCR, *PlxnD1* mRNA was minimally expressed by resting naïve FO B cells, but highly expressed by MZ B cells (**Figure 2.8A,B**). Activation of sorted FO B cells with LPS, anti-IgM, and anti-CD40 resulted in dramatically increased (20-30-fold) plexin-D1 expression, suggesting that this gene might play a role in B-cell immune responses (**Figure 2.8B**). Thus in peripheral lymphoid organs, plexin-D1 is constitutively expressed by MZ B cells and is upregulated in FO B cells upon activation. These data suggest that plexin-D1 expression is associated with activated B-cell populations.

***PlxnD1*$^{-/-}$ B cells exhibit normal development and *in vitro* activation profiles in mice engrafted with *PlxnD1*$^{-/-}$ fetal liver cells**

Conventional *plxnD1* gene inactivation in mice results in embryonic lethality thereby prohibiting any study of a fully developed immune system (Gitler, Lu et al. 2004). To determine if the absence of plexin-D1 affects lymphocyte development, lethally-irradiated C57BL/6 CD45.1 mice were reconstituted with either WT or *PlxnD1*$^{-/-}$ fetal liver cells. In this system, only the hematopoietic compartment was derived from *PlxnD1*$^{-/-}$ (CD45.2) animals, thus permitting the examination of this gene's function in blood cells without the confounding issue of its expression in other tissues. Donor and recipient hematopoietic cell populations were readily distinguished based on the expression of the CD45.1 (host) or CD45.2 (donor) allelic markers. Hematopoietic reconstitution of irradiated mice receiving either WT or *PlxnD1*$^{-/-}$ fetal liver was >90% as assessed by flow cytometric analysis of splenocytes (**Figure 2.1A, i**).

We analyzed B-cell development in the bone marrow of *PlxnD1*$^{-/-}$ and WT control mice by the criteria of Hardy, based on expression of B220, CD43, BP1 and HSA (**Figure 2.1A, ii, iii**) (Li, Wasserman et al. 1996). B220^{+}CD43^{hi} cells were separated into three populations: BP-1^{-}HSA^{-}, BP-1^{-}HSA, and BP1^{+}HSA^{+}: A (prepro-B cells), B (pro-B cells), and C (late pro-B cells). BM of *PlxnD1*$^{-/-}$ and WT control animals showed no difference in the percent of pro-B cells and pre-B cells (**Figure 2.1A, iii**). These data demonstrate that expression of plexin-D1 is not required to generate early progenitor B cell compartments.

To evaluate B-cell development and maturation, we examined the splenic B-cell compartments of *PlxnD1*$^{-/-}$ and WT control animals using mAbs to B220, IgM, IgD, CD21, CD23, and CD93. The immature transitional B cell compartments, defined by IgM, CD23 and CD93 expression (Wilker, Kohyama et al. 2008), were similar in *PlxnD1*$^{-/-}$ and WT animals, suggesting that B cell development in the spleen is normal (**Figure 2.9A**). FO B cell

($IgM^{int}IgD^{hi}$) frequencies were also comparable between *PlxnD1*$^{-/-}$ and WT control animals (**Figure 2.1A, iv**). In contrast, when MZ B cells were examined, there was a 2-fold reduction in the percentage of these cells in *PlxnD1*$^{-/-}$ mice compared to WT controls while FO B cell percentages remained unchanged, thus, suggesting a potential importance of plexin-D1 in MZ function (**Figure 2.1B**). In addition, we examined the B cell compartment in the peritoneal cavity of *PlxnD1*$^{-/-}$ animals and no differences were found in the percentages and numbers of B1 and B2 compartments (**Figure 2.9B**)

The proliferation of *PlxnD1*$^{-/-}$ B cells in response to anti-IgM or LPS stimulation as determined by ^{3}H thymidine incorporation (**Figure 2.1C**) or CFSE dilution (**Figure 2.1D**) was normal in *PlxnD1*$^{-/-}$ FO and MZ B cells. Histological studies of the spleen revealed normal splenic architecture in *PlxnD1*$^{-/-}$ mice as determined by characteristic white and red pulp structure (**Figure 2.1E**). These data suggest that plexin-D1 is dispensable for B-cell proper localization in the spleen.

***PlxnD1*$^{-/-}$ animals have attenuated germinal center responses.**

The reduction in the percentage of MZ B cells in *PlxnD1*$^{-/-}$ animals compared to WT led us to investigate whether humoral immune responses were affected by the absence of plexin-D1. We analyzed basal Ig levels in WT and *PlxnD1*$^{-/-}$ sera by ELISA. Basal total IgM and IgG_3 were comparable between WT and *PlxnD1*$^{-/-}$ animals but IgG_1 and IgG_{2b} levels were significantly reduced in *PlxnD1*$^{-/-}$ mice compared to WT mice (**Figure 2.1F**).

The defect in TD Ig class switching could be attributed to impaired GC reactions in *PlxnD1*$^{-/-}$ animals. GCs are pivotal sites for facilitating Ig class switching and we analyzed, therefore, the formation of GCs in the spleens of *PlxnD1*$^{-/-}$ and control mice after NP-CGG

immunization (Jacob and Kelsoe 1992). Spleens were harvested on day 14 post-immunization and splenocytes were analyzed by flow cytometry to determine the frequency of $B220^{hi}GL7^{hi}$ GC B cells (Arnold, Campbell et al. 2007) present within the total $B220^{+}$ population. While there was no difference at naïve state, *PlxnD1*$^{-/-}$ animals showed a lower percentage of GC B cells when compared to WT post immunization (**Figure 2.2A**). We observed a two-fold reduction in the percentage of GC B cells for *PlxnD1*$^{-/-}$ mice, at days 7, 14 and 21, compared to WT ($p<0.01$) (**Figure 2.2B**). There was no significant difference in the percentages of $B220^{hi}GL7^{+}$ B cells between WT and *PlxnD1*$^{-/-}$ mice at day 4, suggesting that the kinetics of GC onset were not changed by plexin-D1 (**Figure 2.2B**). Splenic cryosections were labeled with mAbs to TCRb, B220 and GL-7 to determine whether GCs form in *PlxnD1*$^{-/-}$ mice. *PlxnD1*$^{-/-}$ animals showed a reduction in the size of the GCs as determined by histology at Day 14 (**Figure 2.2C**). B cell contribution to GC formation was entirely of donor origin (**Figure 2.10A**). Additionally, we stained splenic sections with CD21 to determine the dark and light zone of the GCs in WT and *PlxnD1*$^{-/-}$ animals (**Figure 2.10B**). Our data demonstrate that dark and light zones are present in both WT and *PlxnD1*$^{-/-}$ mice despite the size of the GCs.

To expand the analysis of GC frequency in WT and *PlxnD1*$^{-/-}$ mice, 5-8 non-serial sections were prepared from immunized spleen samples (**Figure 2.3A**). The number of GCs were counted and normalized to the number of follicles per section to account for differences in area. These results showed that the number of follicles that contained GCs was lower in the *PlxnD1*$^{-/-}$ animals compared to WT controls (**Figure 2.3B**). To quantify the difference in GC size between *PlxnD1*$^{-/-}$ and WT animals, the area of individual GCs was measured using ImageJ software (**Figure 2.3C**). Whereas WT animals showed a higher distribution of the

medium and large GCs, *PlxnD1*$^{-/-}$ mice contained an elevated frequency of small GCs and a reduced frequency of large GCs (**Figure 2.3D**). A compilation of >200GC/mouse type shows that the size of GCs in *PlxnD1*$^{-/-}$ mice is reduced by approximately 2-fold compared to the WT controls (**Figure 2.3E**). Taken together these data demonstrate that hematopoietic-derived plexin-D1 controls both the number and size of GCs formed during immunization.

The NP-hapten elicits a restricted antibody response to Igh^{b}/l1 mice (Rajewsky 1996). To exclude the possibility that the differences we observed in the GC reaction are a result of different frequencies of Igλ^{+} B cells in the absence of plexin-D1, we enumerated Igλ^{+} B cells in *PlxnD1*$^{-/-}$ mice and found comparable percentages and distributions of Igλ^{+} B cells to WT (**Figure 2.11A, B**). We also determined the presence of Igλ^{+} cells in the GCs of WT and *PlxnD1*$^{-/-}$ mice (**Figure 2.11C**).

***PlxnD1*$^{-/-}$ B cells enter GC less efficiently than WT B cells.**

To determine whether the expression of plexin-D1 was required for optimal GC formation, irradiated WT mice were reconstituted with fetal liver cells from *PlxnD1*$^{-/-}$ and WT mice (**Figure 2.4A**). After eight weeks, double chimera mice were immunized with NP-CGG. Fourteen days after immunization spleens from immunized and naïve mice were analyzed by flow cytometry and histology. The reconstitution of irradiated recipients by WT and *PlxnD1*$^{-/-}$ hematopoietic cells were similar, in the fact the latter reconstituted somewhat better (**Figure 2.4B**). We determined the frequency of GC B cells (B220^{hi}GL7^{hi}) in the chimeric mice post immunization. Flow cytometry analysis revealed a near three-fold decrease in the percentage of GC B cells derived from *PlxnD1*$^{-/-}$ fetal liver cells compared to WT (**Figure 2.4B, C**). Results obtained from six individual mice are compiled in **Figure**

2.4C. Additionally, we performed immunofluorescence staining on sections obtained from the spleens of chimeric mice to determine the presence of WT or *PlxnD1*$^{-/-}$ B cells in the GC (**Figure 2.4Di**). We observed a lack of CD45.2^{+} *PlxnD1*$^{-/-}$ B cells in >70% of the GC examined. In a competitive environment, *PlxnD1*$^{-/-}$ B cells are less efficient than WT cells in entering GC. Additionally, we stained splenic sections with CD21 to define the light zone of the GC. We observed abundant CD45.1^{+} WT but only occasional CD45.2^{+} *PlxnD1*$^{-/-}$ B cells in the light zone of the GCs (**Figure 2.4Dii**). The majority of *PlxnD1*$^{-/-}$ B cells accumulated around the GCs. These data are consistent with the results obtained earlier with *PlxnD1*$^{-/-}$ mice to indicate that formation of GC is impaired.

B cell expression of plexin-D1 is needed for optimal GC formation.

To determine whether the defective GC responses observed in *PlxnD1*$^{-/-}$ mice was intrinsic to the B cells compartment, we purified B cells from *PlxnD1*$^{-/-}$ and WT mice and transferred them into B-cell deficient animals (μMT). Ten to fourteen days post B-cell transfer, chimeric animals were immunized with NP-CGG and their spleens were analyzed 14 days later (**Figure 2.5A**). We performed flow cytometry analysis to determine the percentage of GC B cells (B220^{hi}GL7^{hi}) in the chimeric mice post immunization. Our results revealed a greater than 3-fold decrease in the percentage of GC B cells in the spleens of *PlxnD1*$^{-/-}$ mice compared to WT mice (**Figure 2.5B**). These results demonstrate an essential function for plexin-D1 expression in B cells for the optimal formation and maintenance of GCs.

PlxnD1*$^{-/-}$ *B cells exhibit reduced migration to CXCL12, CXCL13 or CCL19.

To investigate possible mechanisms behind the reduced size and frequency of GC in *PlxnD1*$^{-/-}$ mice, the response to B-cell chemokines involved in GC recruitment (CXCL12, CXCL13 and CCL19) was measured using an *in vitro* transwell migration assay performed with activated B cells. It has been suggested that CCL19, CXCL12 and CXCL13 are important in establishing GCs and regulating the migration of B cells within GC. CXCL12 is required for recruitment of B cells into the GC dark zone where they undergo rapid proliferation. CXCL13 is required for recruitment into the light zone, where the GC B cells are thought to be selected and become memory cells (Allen, Ansel et al. 2004).

In addition to the *in vivo* experiment described in **Figure 2.4** where WT and *PlxnD1*$^{-/-}$ B cells were assessed for their movement into the GC in a competitive assay, we performed an analogous experiment where activated WT and *PlxnD1*$^{-/-}$ B cells were combined in an *in vitro* competitive B cell migration assay towards CXCL12, CXCL13 and CCL19. Migration of B cells was quantified by flow cytometry and data are presented as the percent of migrating B cells over input. Migration towards these chemokines was uniformly reduced in *PlxnD1*$^{-/-}$ cells compared to WT B cells in the same wells (**Figure 2.6A**). Another group had previously demonstrated that B cells that lack the receptor for CXCL13, CXCR5, are largely excluded from the GC and these GCs display abnormal morphology (Allen, Ansel et al. 2004). Thus, we also assessed the cell surface expression of receptors for CCL19, CXCL12, and CXCL13 by flow cytometry. We did not observe significant differences in cell surface expression for CCR7, CXCR4, and CXCR5 in naïve or activated B cells (**Figure 2.6B**), indicating that the lack of responsiveness to chemokines was not due to lowered expression of the associated chemokine receptors. These observations demonstrate that plexin-D1 regulates B cell migratory responses to a panel of chemokines important for GC reaction.

***PlxnD1*$^{-/-}$ mice show attenuated secondary humoral immune response.**

Given the impaired GC response of *PlxnD1*$^{-/-}$ chimeras, we examined the generation of humoral immune memory in these mice. The GC reaction is crucial for the efficient production of memory B cells and long-lived BM plasma cells, which comprise the cellular memory compartments (Jacob, Przylepa et al. 1993; Allen, Okada et al. 2007). To measure memory B cell responses, WT and *PlxnD1*$^{-/-}$ mice were immunized with NP-CGG in alum and then boosted with NP-CGG 28 days post primary immunization. Serum samples were collected before immunization, 7, 14 and 28 days post primary immunization and 14 days after secondary boost. An ELISA to determine both total and high affinity serum Ab was performed with all serum samples.

Naïve *PlxnD1*$^{-/-}$ or WT mice contained low to undetectable levels of NP reactive IgG_1 Ab, whereas 14 days post immunization NP specific Ab levels in these mice increased (~1000-fold) (**Figure 2.7A**). Primary serum Ab responses of *PlxnD1*$^{-/-}$ and WT animals did not differ ($p>0.05$) in high or total affinity antibody levels (**Figure 2.7A, 7B**). Secondary Ab responses, however, were significantly reduced ($p<0.05$) in *PlxnD1*$^{-/-}$ mice compared to WT for both low and high affinity antibody (**Figure 2.7A, 7B**). These results showed that the initial immune response in *PlxnD1*$^{-/-}$ mice is similar to WT controls while a secondary challenge revealed that the recall response is sub-optimal in *PlxnD1*$^{-/-}$ mice.

To examine whether antigen-specific class switching AFCs were affected in *PlxnD1*$^{-/-}$ animals, we performed ELISPOT assays to determine the frequencies of NP-specific IgG secreting cells in the spleens of immunized mice at 5 and 14 days after primary immunization. The number of splenic NP-specific antibody forming cells (AFC) increased after immunization compared to naïve controls in both WT and *PlxnD1*$^{-/-}$ animals. However,

the frequency of NP-specific IgG AFC was lower in *PlxnD1*$^{-/-}$ animals compared to WT animals at day 14 but not at day 5 after primary immunization (**Figure 2.7C**). Interestingly, we did not observe a difference in the numbers of NP-specific IgM AFC at day 5 and 14 post immunization suggesting that formation of non-class-switched plasma cells is normal in *PlxnD1*$^{-/-}$ animals (**Figure 2.12**). Taken together these data suggest that initial extrafollicular responses in *PlxnD1*$^{-/-}$ mice are intact. We also compared the levels of NP-specific AFC in the BM of *PlxnD1*$^{-/-}$ or WT control animals following a secondary boost where long-lived, memory AFC reside. *PlxnD1*$^{-/-}$ mice exhibited a reduction of NP-specific IgG AFCs following secondary challenge (**Figure 2.7D**). We correlate a reduction in the frequency of NP-specific AFC with reduced levels of IgG_1 observed at day 42, which is 14 days after the secondary challenge.

Taken together these data suggest that although initial AFC formation is normal in *PlxnD1*$^{-/-}$ mice, the continued generation of these cells is reduced as compared to WT controls.

We also examined the role of plexin-D1 during a T_i immune response by immunizing *PlxnD1*$^{-/-}$ and WT mice with NP-Ficoll (4-hydroxy-3- nitrophenylacetyl-ficoll) in PBS. There were no significant differences in the levels of NP-specific serum IgM between WT and *PlxnD1*$^{-/-}$ mice in response to NP-Ficoll (**Figure 2.13**). Thus, plexin-D1 expression on B cells appears to specifically regulate GCs and the formation of humoral immune memory associated with T_d responses.

2.4 DISCUSSION

This paper identifies a novel role for plexin-D1 in B-cell biology and represents the first demonstration of a plexin molecule that affects GC formation and humoral immune memory. In our study, *PlxnD1* was expressed at low levels by FO B cells and substantially induced by LPS, IgM or CD40 engagement. Despite its relative abundance in activated B cells, plexin-D1 was not required for FO B cell development and maturation as all B cell subpopulations tested were present in normal frequencies in the bone marrow, spleen, peritoneal cavity, and lymph nodes of *PlxnD1*$^{-/-}$ animals. The enhancement of *PlxnD1* expression by B cells following activation suggested a role for this molecule during the course of an immune response. Indeed, immunization with T_d antigens resulted in a reduced memory IgG_1 response in *PlxnD1*$^{-/-}$ mice compared to the WT mice, accompanied by reduced size and numbers of splenic GCs in *PlxnD1*$^{-/-}$ animals compared to WT. The GC is a primary site for class switch recombination, affinity maturation, and plasma cell and memory cell development (Jacob, Kassir et al. 1991; Liu, Zhang et al. 1991; Arnold, Campbell et al. 2007). The reduced percentage of GC B cells was followed by a significant reduction in antigen specific AFC formation in the BM following secondary challenge. The size and frequency of GCs are important in providing a pool of B cells that develop into AFC producing class-switched Ab (Arnold, Campbell et al. 2007). Plexin-D1 is important in controlling these events and, thereby, ensures robust humoral responses. Plexin-D1 may be important in specifically regulating either the generation or maintenance of memory B cells. Both of these events are needed to provide a robust memory immune response and defects in either compartment can result in ablated antibody responses (Jacob, Kelsoe et al. 1991;

Kelsoe 1996). Alternatively, plexin-D1 may directly regulate GC B cell survival or proliferation, affecting the size and number of GCs.

Early T_i immune responses in *PlxnD1*$^{-/-}$ animals are not affected, in the context of the NP-FICOLL response, despite a reduction in the number of MZ B cells. This suggests differential requirements for plexin-D1 during the course of an *in vivo* immune response controlled by different types of B cells. These data resemble the phenotype observed in *Rap1b*$^{-/-}$ animals where the MZ B cell compartment is reduced due to the role of *rap1b* in controlling integrin functions (Chen, Yu et al. 2008). However, *Rap1b*$^{-/-}$ mice exhibit unaltered follicular B cell compartments. MZ B cells can participate in the TD immune response by acting as antigen presenting cells and shuttling in and out of the follicles (Martin and Kearney 2002). A reduction in MZ B cell numbers can result in altered TD responses. However, assessment of early TD responses in *PlxnD1*$^{-/-}$ suggest that the phenotype observed in the absence of plexin-D1 is probably not due to the reduced numbers of MZ B cells. Given the emergence of post-GC, antigen-specific and IgG class-switched B cells, we suggest that the smaller GC size in the *PlxnD1*$^{-/-}$ mice may result in a smaller pool of memory B cells generated during the primary response, rather than a defect in the ability to produce memory B cells.

Defects in the GC reaction can be the result of abnormal structural organization of lymphoid tissues (Matsumoto, Lo et al. 1996). For example, *LT*$\alpha^{-/-}$ mice have disturbed splenic architecture due to the inability of T and B cells to form distinct T-and B-cell zones (De Togni, Goellner et al. 1994). This phenomenon leads to unsuccessful IgG responses in these mice following immunization (De Togni, Goellner et al. 1994). Immunohistochemistry

analysis of *PlxnD1*$^{-/-}$ spleens revealed normal splenic architecture, suggesting that the separation of B and T cells into distinct compartments is unaffected in these animals.

Plexins were originally identified in the nervous system as receptors for semaphorins, a large class of both secreted and membrane-bound glycoproteins (Kruger, Aurandt et al. 2005). Plexins are grouped into four different families (A, B, C, and D) based on their structural homology (Kruger, Aurandt et al. 2005). Semaphorins are divided into eight subclasses; classes 1 and 2 belong to invertebrates, classes 3-7 belong to vertebrates and class 8 belongs to viruses. Semaphorins can bind to their primary receptors, plexins, alone or in the presence of neuropilin receptors. Plexins control cell adhesion via their intracellular domains, which contain GTPase-binding structures and are responsible for an array of signaling cascades (Sakurai, Gavard et al. ; Oinuma, Ishikawa et al. 2004). Absence of plexins can lead to impaired immune responses controlled by various cell types (Granziero, Circosta et al. 2003; Wong, Brickey et al. 2003; Walzer, Galibert et al. 2005; O'Connor, Eun et al. 2008; Yamamoto, Suzuki et al. 2008).

The importance of plexin-D1 in cell adhesion and migration has been demonstrated in both, the vascular and immune system. Absence of plexin-D1 results in aberrant vasculature patterning and growth via mechanisms that involve R-Ras and Arf6 (Sakurai, Gavard et al. ; Gu, Yoshida et al. 2005). In the immune system, absence of plexin-D1 results in impaired activated thymocyte migration towards CXCL12 and CCL23 chemokines (Choi, Duke-Cohan et al. 2008). We extend these studies to show a role for plexin-D1 in B cell migration in peripheral lymphoid organs. *In vivo*, lack of plexin-D1 results in impaired B cell migration into the GC. *In vitro*, migration of plexin-D1 deficient B cells towards chemokines that are implicated in T_d immune responses as well as the GC reaction is impaired. CCL19 has been

implicated in recruiting B cells to the spleen and further contributing to their ability to travel and scan different areas in search for their cognate antigen (Okada, Ngo et al. 2002; Okada, Miller et al. 2005). Additionally, CXCL13 is highly expressed in the spleen and is required for migration of B cells to the follicles and subsequently to nascent GCs (Forster, Mattis et al. 1996; Allen, Ansel et al. 2004). CCL19, CXCL12 and CXCL13 are thought to be important in setting up GCs and additionally regulating recruitment of B cells to the LZ or DZ (Allen, Ansel et al. 2004). Our *in vitro* migration experiments revealed that activated B cells had a reduction in migration abilities towards the tested chemokines, CXCL12, CXCL13 and CCL19. This occurred despite the fact that the receptors for these chemokines were expressed at normal levels by *PlxnD1*$^{-/-}$ B cells. These findings suggest that plexin-D1 may be important in allowing activated B cells to enter the GC *in vivo* by facilitating effective migration towards GC chemokines. We have identified a novel mechanism regulating B cell migration to GC chemokines that is distinct from chemokine receptor expression. The mechanisms underlying the ability of plexin-D1 to specifically regulate migration of GC B cells to CXCL12 CXCL13 and CCL19 will be examined in future studies.

The importance of plexins and semaphorins in controlling immune cell development and immune responses is recently being appreciated. For example, Plexin-B1 is required for proliferation and survival of B cells (Granziero, Circosta et al. 2003). Plexin-A1 expression by DCs is needed for proper T-cell activation and proliferation via control of rho activation and actin polarization events in DCs (Wong, Brickey et al. 2003; Eun, O'Connor et al. 2006). Plexin-A4 is a negative regulator of T cell immune responses and its absence leads to development of autoimmunity in mice lacking plexin-A4 (Yamamoto, Suzuki et al. 2008). Most recently, expression of plexin-B2 in B cells was used to identify GCs (Yu, Cook et al.

2008). In this study, we have identified plexin-D1 as a novel regulator of humoral immune memory. Some of the defects observed over the course of a T_d immune responses could be attributed to the inability of activated *PlxnD1*$^{-/-}$ B cells to migrate to the GC. These studies show that a single member of the plexin family can exert various functions on different cell types (T and B) depending on the stage of cell development or the state of immune activation. Further studies involving *PlxnD1*$^{-/-}$ T cells and B cells are needed to define the role of this molecule in different types of immune responses. Plexin-D1 is the first plexin shown to be directly involved in the control of GCs and consequently control of humoral immune memory. This finding has implications in vaccine design in terms of targeting the GC and promoting long-term immunity.

2.5 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 generous gift from Dr. Thomas Jessell's laboratory and have been described in (Gu, Yoshida et al. 2005). μMT mice (*Igh-6*^{tm1Cgn}) 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*$^{+/-}$ mice were crossed for over 10 generations with C57BL/6 mice and intercrossed to obtain *PlxnD1*$^{-/-}$ and *PlxnD1*$^{+/+}$ embryos. *PlxnD1*$^{+/+}$ and *PlxnD1*$^{-/-}$ fetal livers were prepared from E14 embryos post PCR genotyping as previously described (Gu, Yoshida et al. 2005). Five million fetal liver cells were then injected (iv) into lethally irradiated C57BL/6 CD45.1 mice. Mice were analyzed 6-10 weeks post reconstitution. For mixed chimera experiments fetal liver cells were obtained from *PlxnD1*$^{-/-}$ embryos (CD45.2) and *PlxnD1*$^{+/+}$ embryos (CD45.1), mixed (1:1 ratio) and injected into lethally irradiated C57BL/6 CD45.1 recipient mice. Mice were allowed to reconstitute for 6-8 weeks before use.

For μMT experiments, B cells were isolated form *PlxnD1*$^{-/-}$ and *PlxnD1*$^{+/+}$ mice and 2 x 10^7 to 4 x 10^7 mice were injected intravenously into μMT animals. Mice were allowed to reconstitute for 10 days and were then used for experiments.

Immunizations.

NP-CGG immunization: Fetal liver chimeric mice were immunized (ip) with NP_{18}-CGG (4-hydroxy-3- nitrophenylacetyl$_{18}$-chicken gamma globulin) (10mg) precipitated in alum and suspended in 200ml PBS. Mice were bled before, 4, 7, 14 and 28 days after immunization to determine antigen-specific serum Ab levels. Spleens were harvested 4, 7, 14 and 21 days post-immunization and analyzed via FACS and immunofluorescent labeling of tissue sections. Remaining animals were boosted with NP_{18}-CGG (10 mg) at 28 days post primary immunization. Serum was then collected 14 days post boost. *NP-Ficoll immunization:* Fetal liver chimeric animals were immunized (ip) with NP-Ficoll (25mg) suspended in 200ml PBS. Mice were bled before, 3 and 5 days after immunization to determine antigen-specific serum Ab levels.

ELISA

ELISA plates (BD Falcon) were coated with 2μg/ml (50μl/well) of capture reagent goat anti-mouse $Ig_{(H+L)}$, NIP_2-BSA or NIP_{25}-BSA in 0.1M Carbonate Buffer (pH9.5) overnight at 4°C. Detection antibodies included HRP-conjugated goat anti-mouse IgM and goat anti-mouse $IgG_{1, 2b}$, and $_3$ from Southern Biotechnology Associates (Birmingham, AL). Each serum sample was diluted 1:333 and was followed by 5-10 serial 3-fold dilutions and assayed in duplicate. Purified mouse IgM and IgG mAb were used to generate standard curves beginning at 1μg/ml and diluted to 6 ng/ml.

***In vitro* B-cell migration**

B cells were sorted form the spleens of WT and *PlxnD1*$^{-/-}$ animals and suspended in RPMI 1640 with 0.1% fatty acid free BSA. Cell were resuspended at a density of 10^7 cells

per ml and incubated at 37°C for 30 minutes prior to transwell migration assay. Media (100ml) was added to the upper chamber of a 24-well migration plates with a pore size of 5mm (Corning Costar). Top chambers were inserted into wells containing 600μl of media with CCL19 (500ng/ml), CXCL13 (500ng/ml) or CXCL12 (150ng/ml). Plates were incubated for 2hrs at 37°C. All cells were counted by flow cytometry as previously described (Allen, Ansel et al. 2004).

ELISPOT

Antigen-specific AFC: ELISPOT plates (Millipore) were coated with 2μg/ml (50 μl/well) of NP_{25}-BSA or goat anti-mouse $Ig_{(H+L)}$ in 0.1M Carbonate Buffer (pH9.5) overnight at 4°C. One million splenocytes (5d and 14d post primary immunization) and 1 million BM cells (14d post boost) obtained from immunized animals were serially diluted and plated in triplicate. Cells were incubated at 37°C in a humidified CO_2 incubator for 4h. Plates were washed and re-blocked for 24hrs using blocking buffer (1xPBS (pH7.4), 0.1% Tween-20 and 0.5% BSA). Membranes were probed with AP-conjugated IgG (Southern Biotechnology Associates, Birmingham, AL) and spots were developed with SIGMA *FAST* BCIP/NBT (Sigma).

Cell culture

Proliferation assays: B cells were sorted by FACS or using a B-cell negative selection kit (Stemcell Technologies). $2x10^5$ purified B cells were cultured in RPMI media (Gibco) containing 10% FBS, 10^{-4}M 2-ME and penicillin/streptomycin (P/S) antibiotics in 96 well plates. Cells were cultured in the presence of 5 μg/ml LPS (SIGMA) or 5μg/ml anti-μ

(Jackson ImmunoResearch) for 48 hours. One mCi of ^{3}H thymidine was added to the cultures for the last 8 hours to assess proliferation. FACS sorted FO ($B220^{+}CD21^{lo}CD23^{hi}$) B cells were labeled with 5mM CFSE (Sigma) and cultured in the presence of 5μg/ml IgM or 5μg/ml LPS. Proliferation was assessed by flow cytometry after 72 hours. *B cell activation:* FO and MZ B cells were sorted from the spleens of C57BL/6 animals and cultured in the presence of 5 μg/ml LPS (SIGMA), 1mg/ml anti-m (Jackson ImmunoResearch), or 1μg/ml anti-CD40 (eBiosciences) for 72 hours. RNA and cDNA for RT-PCR and quantitative real-time PCR were prepared as described above.

Antibodies and FACS

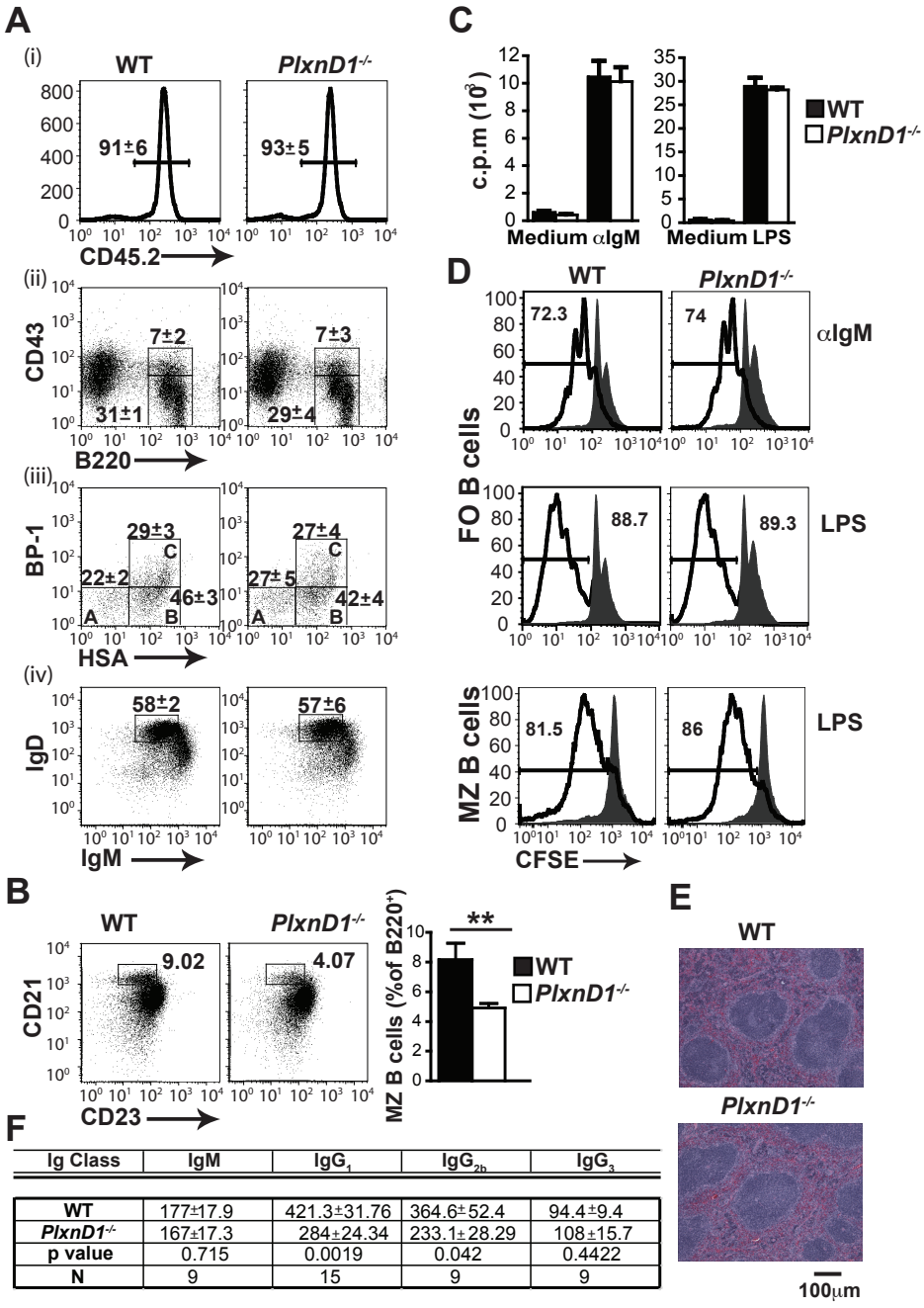
Monoclonal Abs included: B220 (RA3-6B2), CD23 (B3B4), BP-1 (6C3), CD24 (M1/69), CD43 (S7), GL-7 (GL-7) and APC-Alexa750-conjugated streptavidin from BD Pharmingen (San Diego, CA); IgM (eB121-15F9), IgD (11-26), CD21 (eBio8D9), CD93 (AA4.1), CD5(Ly-1), CD45.2 (104), CD4 (L3T4), CD8 (Ly-2), and TCRβ (H57-597) from eBioscience (San Diego, CA); Igκ, Igλ from Southern Biotech (Birmingham, AL); CD38 (90) and IgG1 (RMG1-1) from Biolegend (San Diego, CA). Secondary antibodies included anti-FITC-Alexa488, Alexa350-conjugated streptavidin and Alexa405-conjugated streptavidin from Invitrogen (Raleigh, NC). 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 or LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting was performed on a MoFlo cytometer (Beckman-Coulter).

Histology

Spleens from naïve and immunized mice were embedded in OCT compound, snap frozen, and stored at -80°C. 5 μm sections were prepared and fixed with 1:1 Acetone:Methanol for 10 min at -20°C and labeled with various combinations of B220-biotin, B220-FITC (green), B220-PE (red), TCRβ-PE (red), GL-7-FITC (green), CD21-biotin, Igλ-FITC (green), Igκ-biotin, CD45.1-FITC, -PE, or –biotin, and CD45.2-FITC, -PE, -Biotin mAb. FITC signal was amplified using anti-FITC-Alexa488 mAb. Streptavidin-AlexaFluor350 or Streptavidin-AlexaFluor405 was used to amplify -biotin signal (blue). Additionally, sections from various spleens were H&E stained. Images were acquired using a Zeiss Axiovert 200M or Zeiss LSM 710 confocal immunofluorescent microscope.

Statistical analysis

Statistical significance was determined with two-tailed Student's t test. All *p* values less than 0.05 were considered significant.



Ig Class	IgM	IgG_1	IgG_{2b}	IgG_3
WT	177±17.9	421.3±31.76	364.6±52.4	94.4±9.4
PlxnD1$^{-/-}$	167±17.3	284±24.34	233.1±28.29	108±15.7
p value	0.715	0.0019	0.042	0.4422
N	9	15	9	9

Figure 2.1. Normal *in vitro* function exhibited by *PlxnD1*$^{-/-}$ B cells

(A) Reconstitution of irradiated recipients with WT and *PlxnD1*$^{-/-}$ fetal liver cells. BM and spleen cells were harvested from WT and *PlxnD1*$^{-/-}$ mice and analyzed by flow cytometry. Monoclonal Ab to CD45.2 was used to label splenocytes and to determine the percent of reconstitution. WT and *PlxnD1*$^{-/-}$ fetal liver cells reconstituted equally well **(i)**. Monoclonal Abs to B220 and CD43 **(ii)**, and HSA and BP1 **(iii)** were used to determine the percentages of B cell progenitors in the bone marrow using Hardy's fractionation **(ii, iii)**. Monoclonal Abs to B220, IgM, and IgD were used to determine the percentage of mature FO B cell population in the spleen **(iv)**. Plots are pre-gated on live CD45.2^{+}B220^{+} lymphocytes. Data are representative of 10 mice for each genotype. Mean percentage ± SEM is indicated for each cell type. **(B)** Monoclonal Abs to CD45.2, B220, CD21, and CD23 were used to determine the percentage of MZ B cell populations in the spleen, WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar). Plots were pregated on B220^{+} splenocytes. Graph represents data pooled from 9 mice in 3 different experiments. **$p<0.01$ **(C)** Proliferation of B cells from mice reconstituted with *PlxnD1*$^{-/-}$ and WT fetal liver cells as assessed by ^{3}H thymidine uptake. WT (solid bar) or *PlxnD1*$^{-/-}$ (open bar) FO B cells were cultured in the presence of 5μg/ml anti-IgM or 5μg/ml LPS for 48 hours. Cells were pulsed with ^{3}H thymidine for the last 8 hours in culture to determine cell proliferation and data are presented as counts per minute (c.p.m). n=9 mice per group. Data are representative of 3 independent experiments. **(D)** Proliferation of *PlxnD1*$^{-/-}$ and WT B cells as assessed by CFSE dilution. FO and MZ B cells were labeled with CFSE and cultured in the presence of 5μg/ml IgM or 5mg/ml of LPS for 72 hours. Proliferation was assessed by flowcytometry and data are representative of 3 independent experiments. n=9 mice per group. **(E)** Splenic structure of mice reconstituted with *PlxnD1*$^{-/-}$

and WT fetal liver cells. H+E staining of 5μm spleen cryosections was performed. Five spleens were analyzed for each genotype. **(F)** Serum Ig levels in mice reconstituted with *PlxnD1*$^{-/-}$ and WT fetal liver cells. Serum was harvested from naïve mice 8 weeks post fetal liver cell transplant. Basal levels of Ab were determined by ELISA using goat anti-mouse $Ig_{(H+L)}$ as capture reagent. Ab was detected using HRP labeled goat anti mouse IgM, IgG_1, IgG_{2b} or IgG_3. Data were pooled from 3 independent experiments. n=9-14 mice per group.

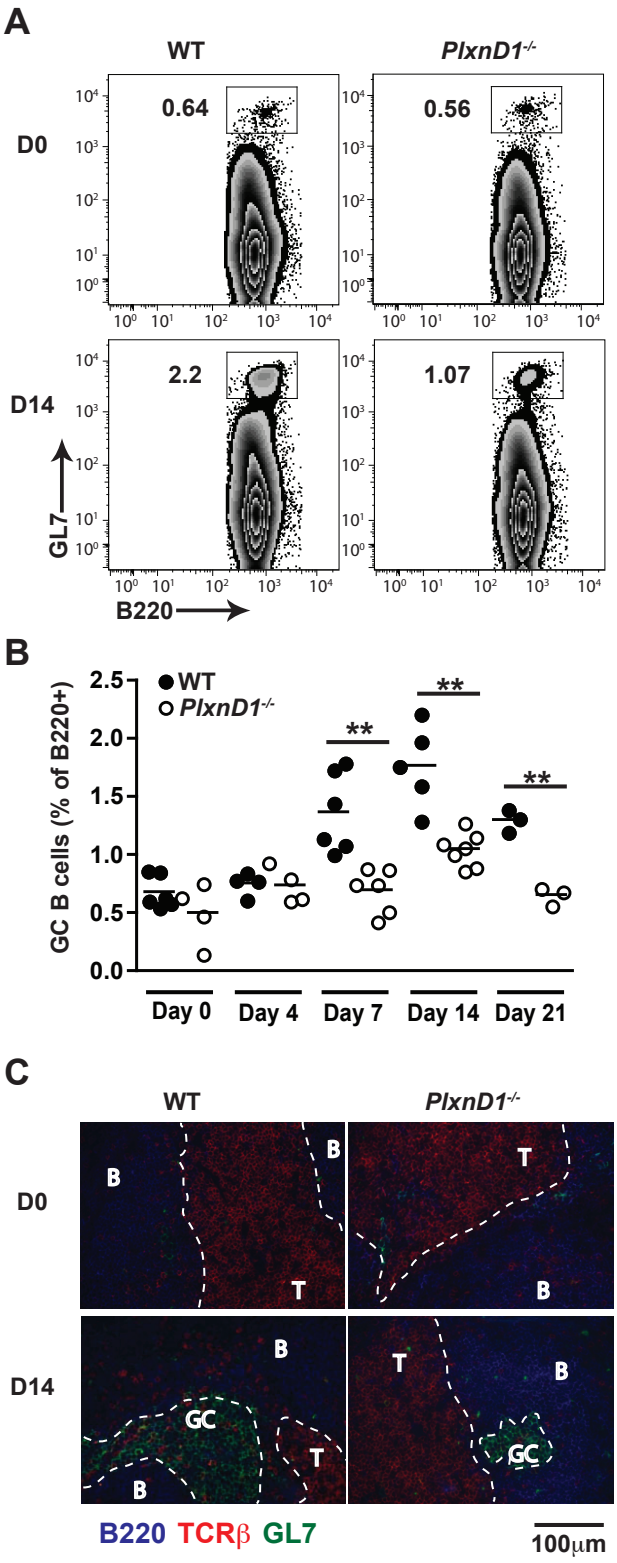
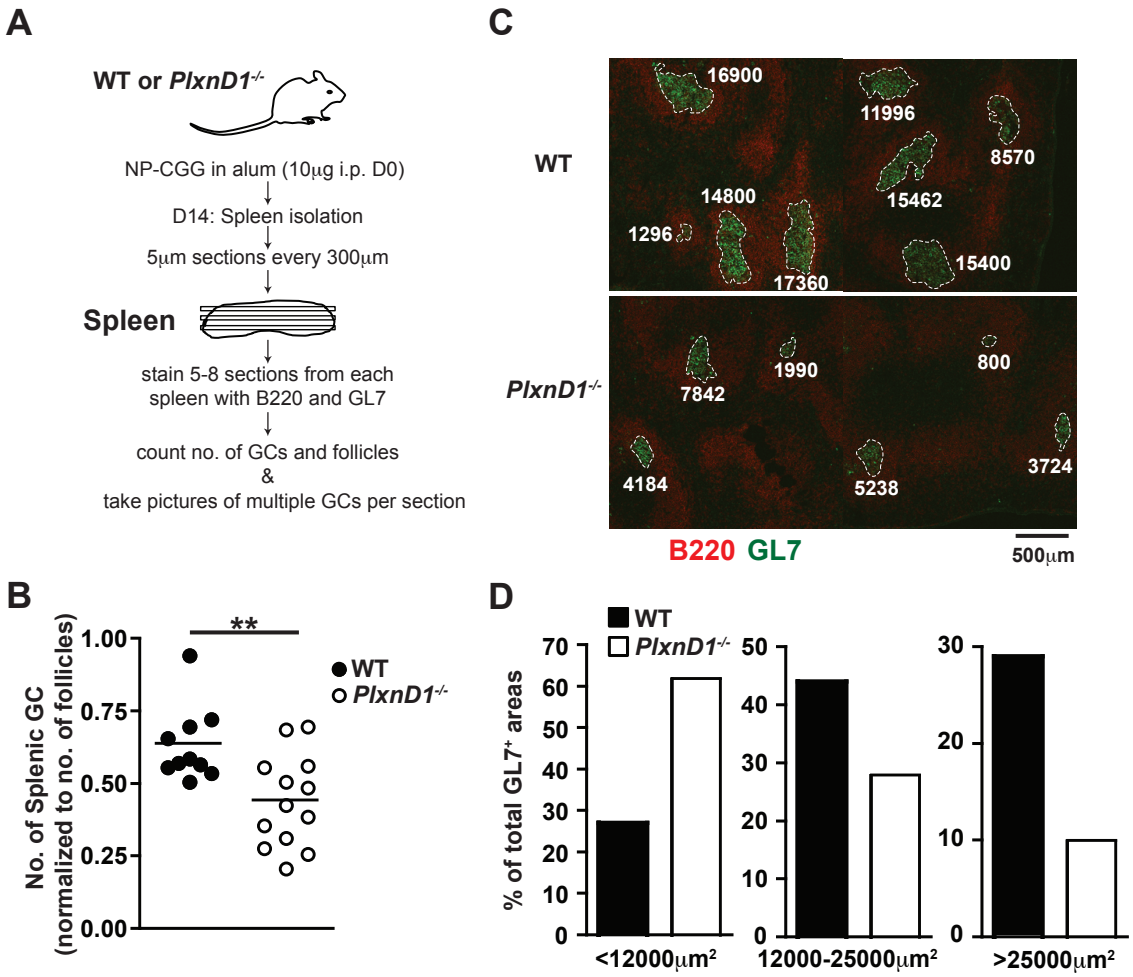

A
WT
PlxnD1-/-
D0
D14
0.64
0.56
2.2
1.07
GL7
B220
B
WT
PlxnD1-/-
GC B cells (% of B220+)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
Day 0
Day 4
Day 7
Day 14
Day 21
C
WT
PlxnD1-/-
D0
D14
B
T
GC
B220 TCRβ GL7
100μm

Figure 2.2. Impaired germinal center response in *PlxnD1*$^{-/-}$ mice.

(A) Reduced frequency of GC B cells in mice reconstituted with *PlxnD1*$^{-/-}$ cells relative to those with WT cells as measure by flow cytometry. Mice were immunized (ip) with 10 mg NP_{18}-CGG in alum and spleens were harvested at day 14 post immunization. Cells were labeled with mAbs to CD45.2, B220, IgM, IgD, TCRβ and GL-7. The percent of B220^{hi}GL-7^{hi} B cells of total B220^{hi} cells was determined by flow cytometry. **(B)** Reduced frequency of GC B cells in *PlxnD1*$^{-/-}$ (open circle) mice relative to WT (closed circle) mice. Quantification of GC B cells as measured by flow cytometry. The graph is representative of 3 independent experiments. Each point represents data from one mouse. Small horizontal bars indicate mean values. **$p<0.001$. **(C)** Reduction of GC size in mice reconstituted with *PlxnD1*$^{-/-}$ cells relative to those with WT cells as measured by histology. Five mm sections of spleens from WT and *PlxnD1*$^{-/-}$ mice at day 14 post immunization were labeled with B220-AF350 (blue), TCRβ-PE (red) and GL-7-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. Day 0 samples are shown as control. n=10 mice per group.



E

Genotype	No. GC analyzed	Median area	Mean area(±SEM)***
WT	206	20449	23840±1247
PlxnD1$^{-/-}$	212	9676	14580±1026

Figure 2.3. Decreased number and size of GCs in *PlxnD1*$^{-/-}$ mice.

(A) Schematic of experimental design. **(B)** Reduced GC number in *PlxnD1*$^{-/-}$ (open circle) mice than WT (solid circle). Number of GCs was counted using 5-8 different 5μm sections for each mouse spleen divided by the total number of B cell follicles. Each point represents data from one mouse. Data were pooled from 3 independent experiments. Small horizontal bars indicate mean values. **p<0.001. **(C)** Reduced size in *PlxnD1*$^{-/-}$ mice than WT. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. Size of individual GCs was determined using ImageJ. **(D)** Reduced percentage of larger GC in mice reconstituted with *PlxnD1*$^{-/-}$ cells (open bar) compared to WT (solid bar). GCs were divided into three groups: small (<12,000μm^2), medium (12,000-25,000 μm^2) and large (>25,000μm^2). The number of GCs in each group was divided by the total number of GCs and presented as a percentage. More than 200 GCs were analyzed for each group. **(E)** Reduced GC area in *PlxnD1*$^{-/-}$ mice. Area of individual GCs was determined 14 days post primary NP-CGG immunization using ImageJ. Data are pooled from three independent experiments. n=13. ***p<0.0001.

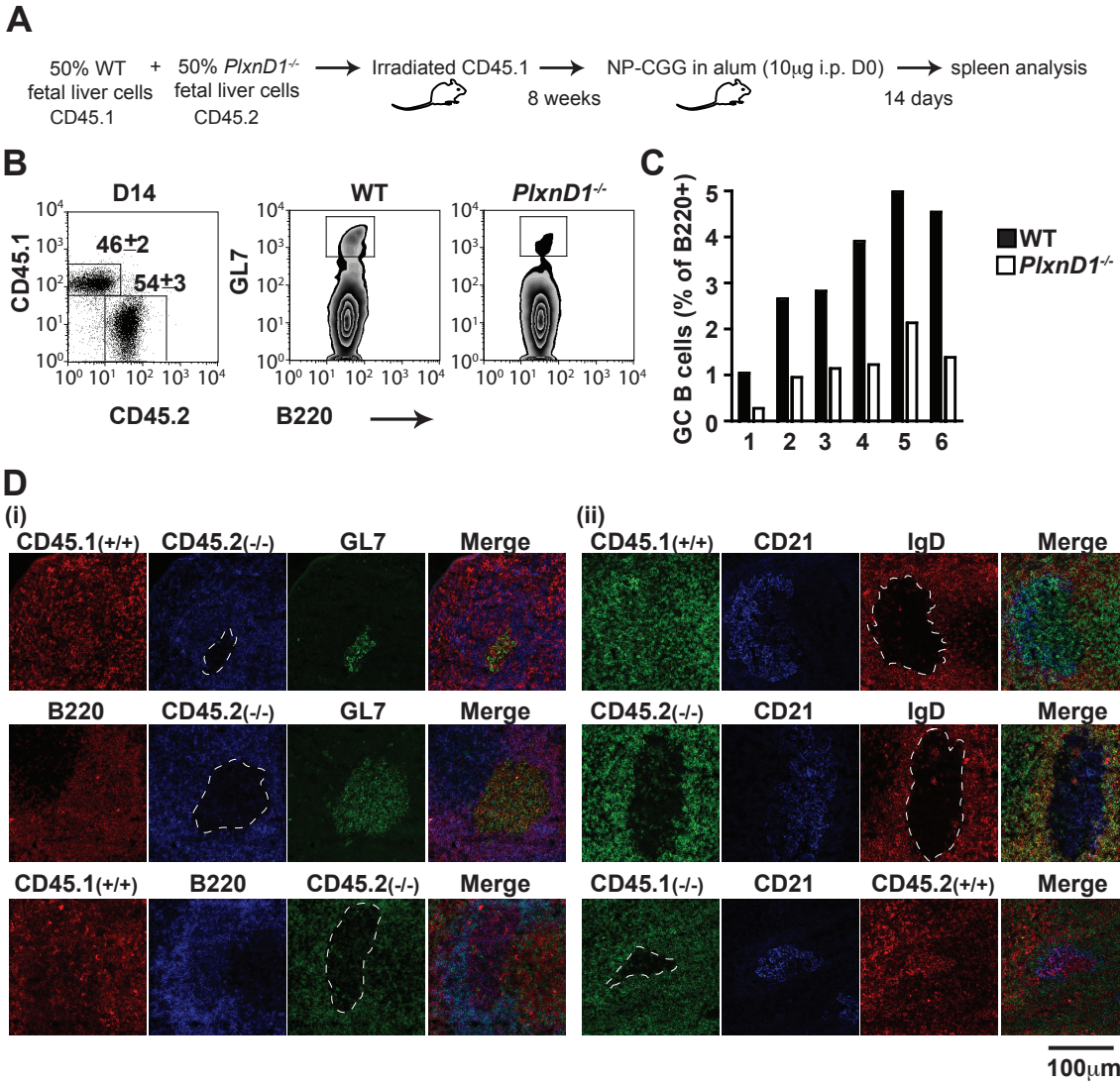

A
50% WT fetal liver cells CD45.1
+
50% PlxnD1-/- fetal liver cells CD45.2
Irradiated CD45.1
8 weeks
NP-CGG in alum (10μg i.p. D0)
spleen analysis
14 days
B
D14
CD45.1
CD45.2
46±2
54±3
WT
PlxnD1-/-
GL7
B220
C
GC B cells (% of B220+)
WT
PlxnD1-/-
D
(i)
CD45.1(+/+)
CD45.2(-/-)
GL7
Merge
B220
CD45.1(+/+)
CD45.2(-/-)
(ii)
CD45.1(+/+)
CD21
IgD
Merge
CD45.2(-/-)
CD45.1(-/-)
CD45.2(+/+)
100μm

Figure 2.4. *PlxnD1*$^{-/-}$ B cells enter GC less efficiently than WT B cells.

(A) Schematic of experimental design. **(B)** Similar hematopoietic reconstitution but reduced frequency of *PlxnD1*$^{-/-}$ derived GC B cells relative to those with WT cells as measured by flow cytometry. Mice were immunized (ip) with NP-CGG in alum and spleens were harvested at day 14 post immunization. Cells were labeled with mAb to CD45.1, CD45.2, B220, IgM, IgD, TCRβ and GL-7. The percent of *PlxnD1*$^{-/-}$ CD45.2^{+}B220^{hi}GL-7^{hi} B cells of total B220^{hi} cells was determined by flow cytometry and was reduced relative to the percentage of WT CD45.1^{+}B220^{hi}GL-7^{hi} controls. n=6 mice per group. Mean±SEM are presented for each genotype. Data were obtained in two separate experiments. **(C)** Reduced frequency of GC B cells in *PlxnD1*$^{-/-}$ (open bars) B cells relative to WT (closed bars) B cells. Quantification of GC B cells as measured by flow cytometry. The graph is representative of 2 independent experiments. Each point represents data from one mouse. **$p<0.001. **(D)** Splenic sections from chimeric mice were stained with various combinations of CD45.1, CD45.2, GL7 and B220 antibodies to determine the presence of WT and *PlxnD1*$^{-/-}$ B cells in the GCs **(i).** Splenic sections from WT/*PlxnD1*$^{-/-}$ chimeric mice were stained with various combinations of CD45.1, CD45.2, IgD, and CD21 antibodies to determine the presence of WT and *PlxnD1*$^{-/-}$ B cells in the GC light and dark zones (ii). Images were acquired using a Zeiss LSM 710 confocal immunofluorescent microscope. n=6 mice per group.

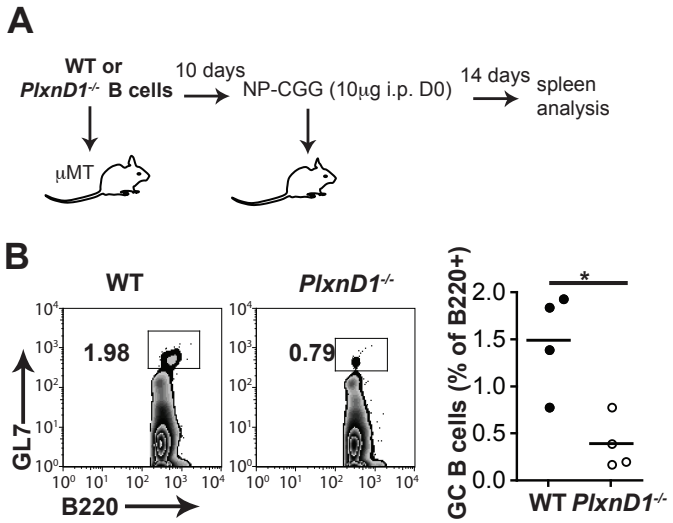

A
WT or
PlxnD1-/- B cells
10 days
NP-CGG (10μg i.p. D0)
14 days
spleen
analysis
μMT
B
WT
PlxnD1-/-
1.98
0.79
GL7
B220
GC B cells (% of B220+)
*
WT PlxnD1-/-

Figure 2.5. Plexin-D1 deficiency in B cells alone results in reduced percentages of GC B cells.

(A) Schematic of experimental design. **(B)** Reduced frequency of GC B cells in B-cell deficient mice (μMT) reconstituted with *PlxnD1*$^{-/-}$ B cells relative to those with WT cells as measure by flow cytometry. Mice were immunized (ip) with NP_{18}-CGG in alum and spleens were harvested at day 14 post immunization. Cells were labeled with mAb to CD45.2, B220, IgM, IgD, TCRβ and GL-7. The percent of B220^{hi}GL-7^{hi} B cells of total B220^{hi} cells was determined by flow cytometry and was reduced in μMT animals reconstituted with *PlxnD1*$^{-/-}$ (open circle) B cells relative to WT (solid cirle) controls. Each circle represents an individual mouse. Data are representative of 2 independent experiments. Small horizontal bars indicate mean values.

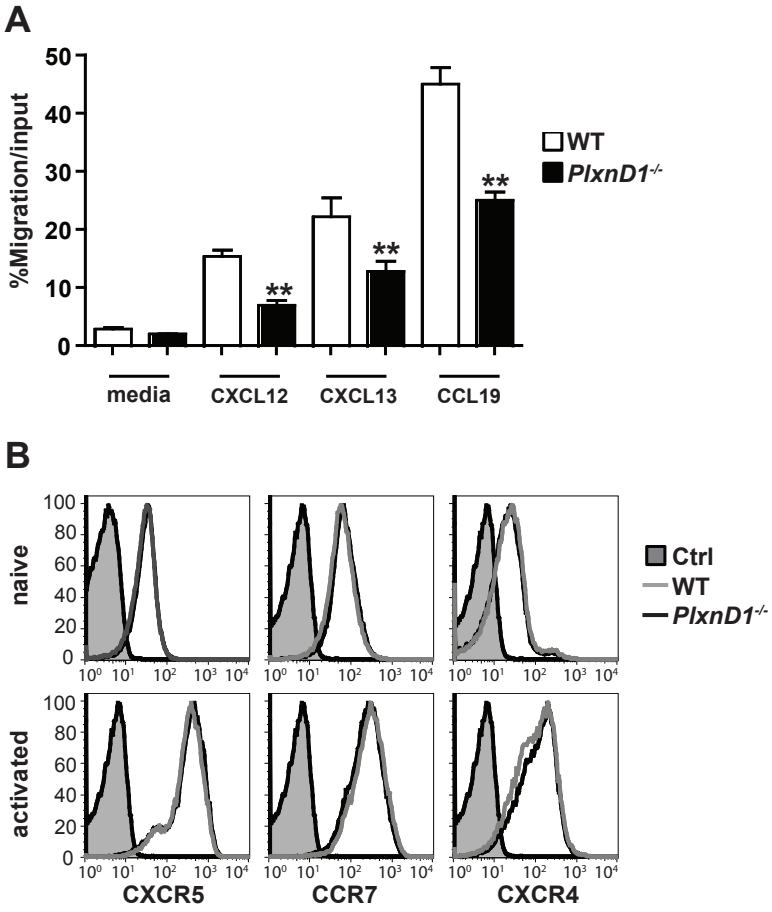

A
%Migration/input
50
40
30
20
10
0
WT
PlxnD1-/-
**
**
**
media
CXCL12
CXCL13
CCL19
B
naive
activated
100
80
60
40
20
0
Ctrl
WT
PlxnD1-/-
CXCR5
CCR7
CXCR4

Figure 2.6. Impaired *in vitro* migration of activated *PlxnD1*$^{-/-}$ B cells but normal surface chemokine receptor expression.

(A) Purified WT (open bar) and *PlxnD1*$^{-/-}$ (solid bar) B cells were placed in the same well and subjected to competitive *in vitro* migration assays in the presence of medium alone, CXCL12, CXCL13 and CCL19 (Figure. 6A). WT and *PlxnD1*$^{-/-}$ B cells were differentiated by CD45.1 and CD45.2 expression. Migrated cells were quantified by flow cytometry and are presented as percent of total input cells. Purified WT and *PlxnD1*$^{-/-}$ were activated in the presence of anti-IgM and anti-CD40 for 24 hours and then subjected to *in vitro* migration assays as described. Data are pooled from three independent experiments. n=6-7 mice per group. **(B)** Spleen cells were harvested from *PlxnD1*$^{-/-}$ (black line) and WT (gray line) mice and analyzed by flow cytometry. Monoclonal Abs to CD45.1, CD45.2 and B220 were used to identify the fetal liver donor B cells and then mAbs to CCR7, CXCR4, or CXCR5 were used to stain these cells **(top).** B cells were isolated from *PlxnD1*$^{-/-}$ and WT spleens and activated with anti-CD40 and anti-IgM for 24 hours **(bottom)**. n=5 mice per group.

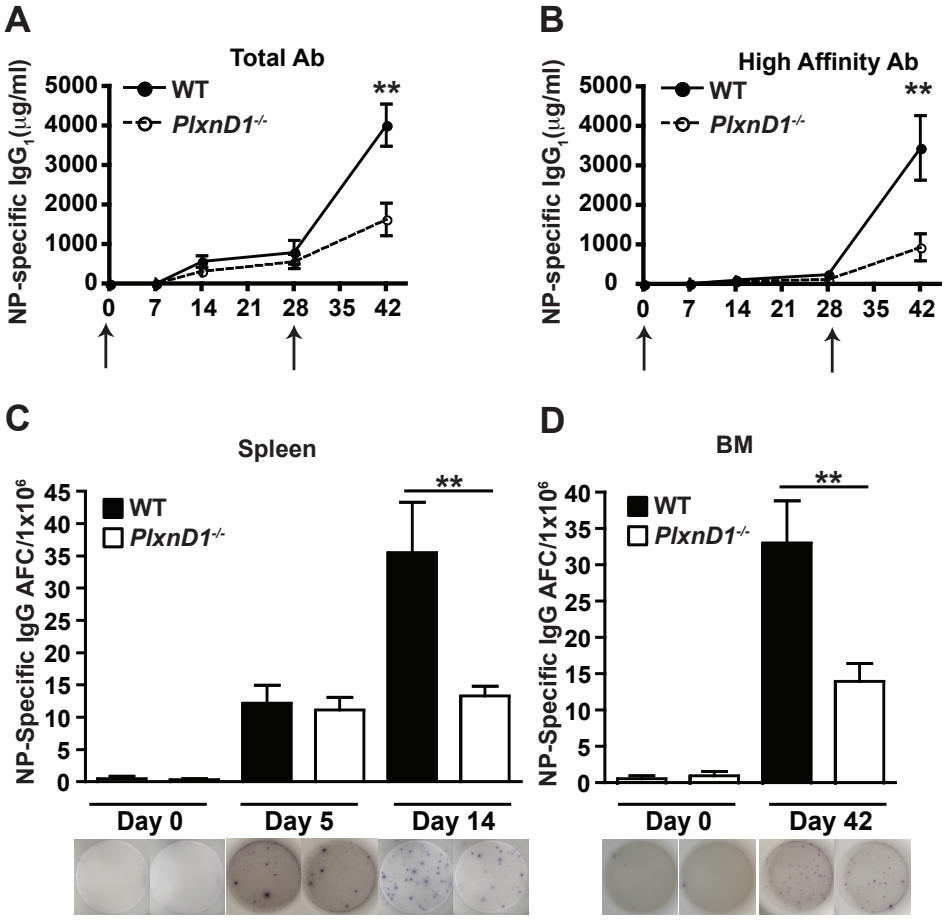

A
Total Ab
NP-specific IgG1(μg/ml)
5000
4000
3000
2000
1000
0
WT
PlxnD1-/-
**
0 7 14 21 28 35 42
B
High Affinity Ab
NP-specific IgG1(μg/ml)
5000
4000
3000
2000
1000
0
WT
PlxnD1-/-
**
0 7 14 21 28 35 42
C
Spleen
NP-Specific IgG AFC/1x10^6
45
40
35
30
25
20
15
10
5
0
WT
PlxnD1-/-
**
Day 0
Day 5
Day 14
D
BM
NP-Specific IgG AFC/1x10^6
40
35
30
25
20
15
10
5
0
WT
PlxnD1-/-
**
Day 0
Day 42

Figure 2.7. Impaired secondary Td immune response in *PlxnD1*$^{-/-}$ mice.

(A, B) Serum Ig levels after primary and secondary immunization in WT (solid line) and *PlxnD1*$^{-/-}$ (dotted line) mice. Mice were immunized (ip) with NP_{18}-CGG in alum and boosted at day 28 post primary immunization. Serum was harvested at various time points. Ab levels were measured by ELISA. Data were pooled from three independent experiments. **$p<0.01$ and n=11 mice per group. **(C)** Antigen specific splenic AFC formation in mice reconstituted with WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar) fetal liver cells. Spleens were harvested at day 5 and 14 post-immunization. NP-specific IgG AFCs were enumerated by ELISPOT on wells coated with NP_{25}-BSA. **(D)** Antigen specific bone marrow AFC formation in mice reconstituted with *PlxnD1*$^{-/-}$ and WT fetal liver cells. Bone marrow was harvested at day 14 post-secondary immunization. NP specific IgG AFCs were enumerated as above. Data were pooled from 3 independent experiments. Representative pictures of ELISPOT wells are shown below each graph. Mean±SEM. **$p<0.01$ and n=10 mice per group.

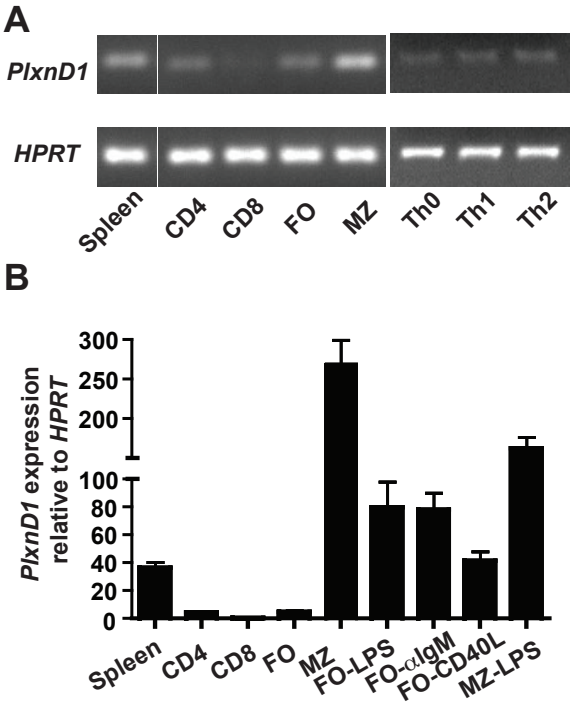

A
PlxnD1
HPRT
Spleen
CD4
CD8
FO
MZ
Th0
Th1
Th2
B
PlxnD1 expression relative to HPRT
300
250
200
100
80
60
40
20
0
Spleen
CD4
CD8
FO
MZ
FO-LPS
FO-αIgM
FO-CD40L
MZ-LPS

Figure 2.8. Expression of plexin-D1 by lymphocytes.

(A) Expression of *PlxnD1* in the spleen, resting CD4 and CD8 T cells, MZ and FO B cells, Th1 and Th2 cells, assessed by RT-PCR. Data are representative of three independent experiments. **(B)** Expression of *PlxnD1* in sorted resting T cells, FO and MZ B cells, and activated B cells as assessed by real-time PCR. All values were normalized to *HPR*T. B cells were cultured in the presence of LPS (5 μg/ml), anti-μ (1μg/ml), or anti-CD40 (1μg/ml) for 72 hours. All splenocytes used for RT-PCR along with real-time PCR were labeled with mAb to CD4, CD8, CD62L, B220, IgM, IgD, CD23 and CD21 and purified by FACS sorting into $CD4^{+}CD62L^{Hi}$ (CD4 T cells), $CD8^{+}CD62L^{Hi}$ (CD8 T cells), $B220^{+}IgD^{Hi}IgM^{Lo}CD21^{Lo}CD23^{Hi}$ (FO B cells), $B220^{+}IgD^{Lo}IgM^{Hi}CD21^{Hi}CD23^{Lo}$ (MZ B cells). Data are representative of three independent experiments. Mean±SEM. n=8 mice.

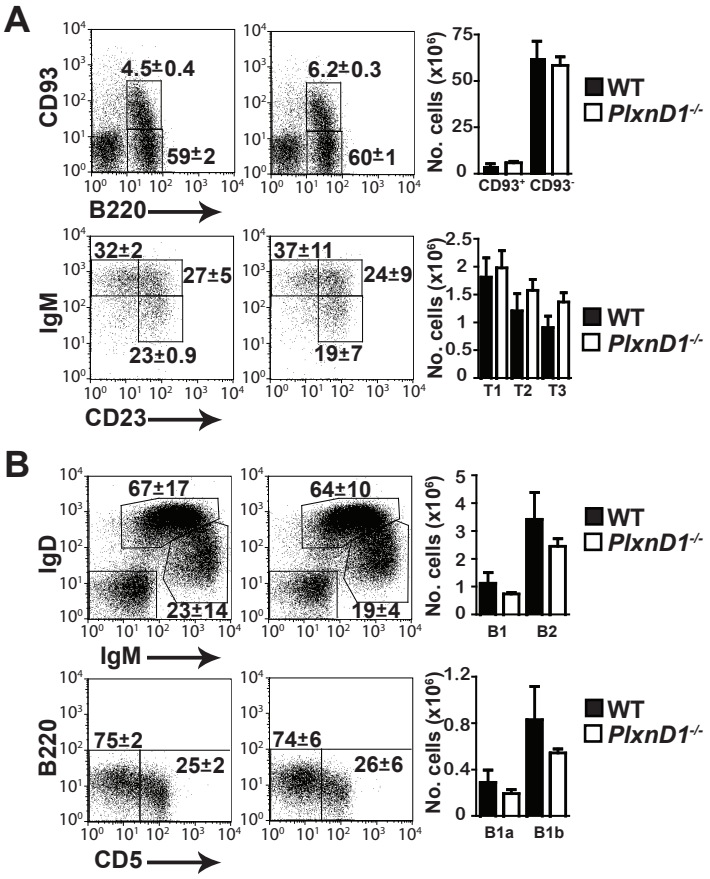

A
CD93
4.5±0.4
59±2
6.2±0.3
60±1
B220
No. cells (x10⁶)
CD93+ CD93-
WT
PlxnD1-/-
IgM
32±2
27±5
23±0.9
37±11
24±9
19±7
CD23
T1 T2 T3
B
IgD
67±17
23±14
64±10
19±4
IgM
B1 B2
B220
75±2
25±2
74±6
26±6
CD5
B1a B1b

Figure 2.9. Normal B cell development in *PlxnD1*$^{-/-}$ mice.

(A) Splenocytes were harvested from WT and *PlxnD1*$^{-/-}$ mice and analyzed by flow cytometry. Monoclonal Abs to B220, CD93, IgM and CD23 were used to determine the percentages of immature (B220^{+}CD93^{+}) and mature (B220^{+}CD93^{-}) splenic B cells among total splenocytes. B220^{+}CD93^{+} cells were then divided into transitional B cell compartments T1, T2, and T3 based on their IgM and CD23 expression. Plots are pre-gated on live CD45.2^{+} splenocytes. Data are representative of 4-5 mice for each genotype. Mean percentage ± SEM is indicated for each cell type. Data are representative of two independent experiments. Total numbers of cells are expressed in the corresponding graphs. WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar). **(B)** Peritoneal cavity lavage cells were harvested from WT and *PlxnD1*$^{-/-}$ mice and analyzed by flow cytometry. Monoclonal Abs to CD45.2, IgD, IgM were used to determine the percentage of B1 and B2 B cell populations in the peritoneal cavity. Further analysis of IgD^{Lo}IgM^{Hi} populations by CD5 was used to separate these cells into B1a (B220^{Lo}CD5^{+}) and B1b (B220^{Lo}CD5^{-}) B cell compartments. Data are representative of 4-5 mice for each genotype. Mean percentage ± SEM is indicated for each cell type. Data are representative of two independent experiments. Total numbers of cells are expressed in the corresponding graphs. WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar).

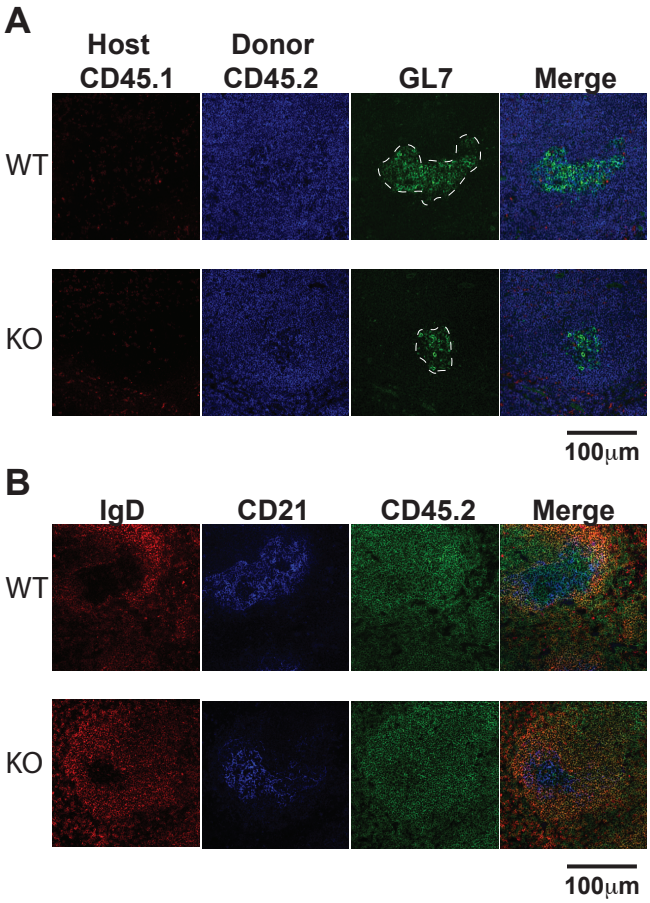

A
Host
CD45.1
Donor
CD45.2
GL7
Merge
WT
KO
100μm
B
IgD
CD21
CD45.2
Merge
WT
KO
100μm

Figure 2.10. B cell contribution in WT and *PlxnD1*$^{-/-}$ GCs.

(A) GCs in both WT and *PlxnD1*$^{-/-}$ mice are made up of donor B cells as measured by histology. Five mm sections of spleens from WT and *PlxnD1*$^{-/-}$ mice at d14 post immunization were labeled with CD45.1 PE (red), CD45.2-AF405 (blue) and GL-7-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. n=5 mice per group**. (B)** GCs in both WT and *PlxnD1*$^{-/-}$ contain light zones and dark zones. Five mm sections of spleens from WT and *PlxnD1*$^{-/-}$ mice at d14 post immunization were labeled with IgD-PE (red), CD21-AF405 (blue) and CD45.2-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. n=5 mice per group. Data are representative of three independent experiments.

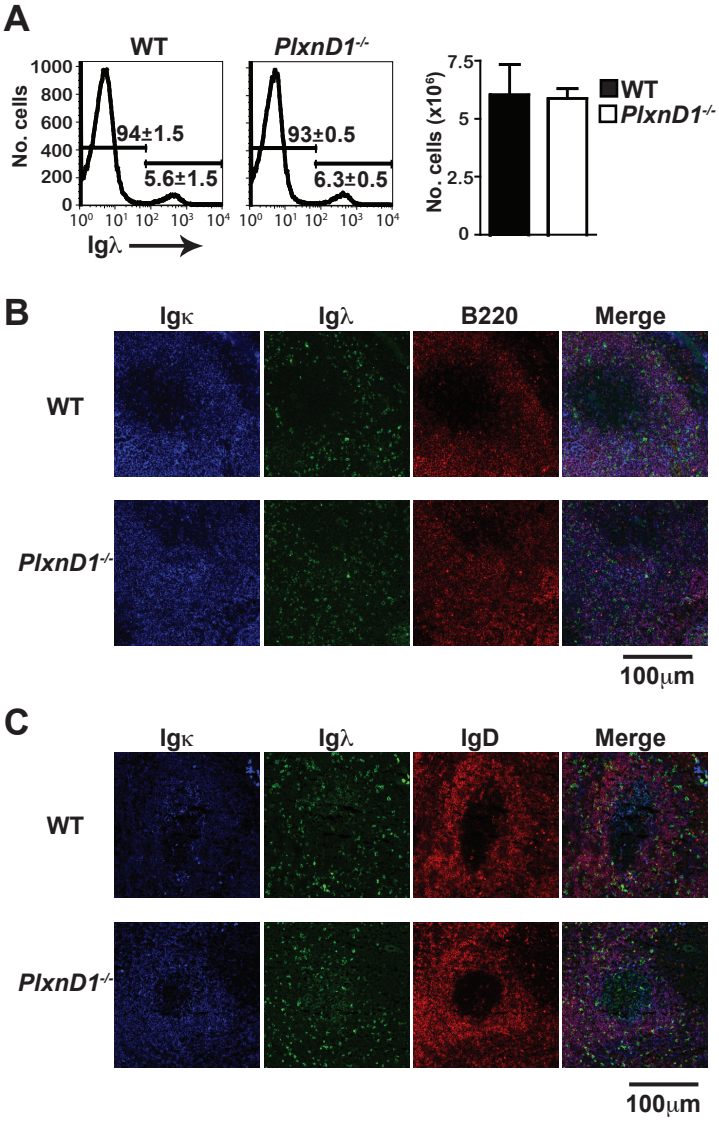

A
WT
PlxnD1-/-
No. cells
94±1.5
5.6±1.5
93±0.5
6.3±0.5
Igλ
No. cells (x10^6)
WT
PlxnD1-/-
B
Igκ
Igλ
B220
Merge
WT
PlxnD1-/-
100μm
C
Igκ
Igλ
IgD
Merge
WT
PlxnD1-/-
100μm

Figure 2.11. Normal Igλ and Igκ B cell repertoire in *PlxnD1*$^{-/-}$ mice.

(A) Splenocytes were harvested from WT and *PlxnD1*$^{-/-}$ mice and analyzed by flow cytometry. Monoclonal Abs to B220, Igλ and Igκ were used to determine the percentages of Igλ and Igκ splenic B cell among total splenocytes. Plots are pre-gated on live CD45.2^{+} splenocytes. Data are representative of 4-5 mice for each genotype. Data are representative of two independent experiments. Mean percentage ± SEM is indicated for each cell type. Total numbers of cells are expressed in the corresponding graphs. WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar). (B) Five mm sections of spleens from WT and *PlxnD1*$^{-/-}$ mice were labeled with B220 PE (red), Igκ-AF405 (blue) and Igλ-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. n=4 mice per group. (C) Five mm sections of spleens from day14 NP-CGG immunized WT and *PlxnD1*$^{-/-}$ mice were labeled with IgD PE (red), Igκ-AF405 (blue) and Igλ-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. n=5 mice per group. Data are representative of two independent experiments.

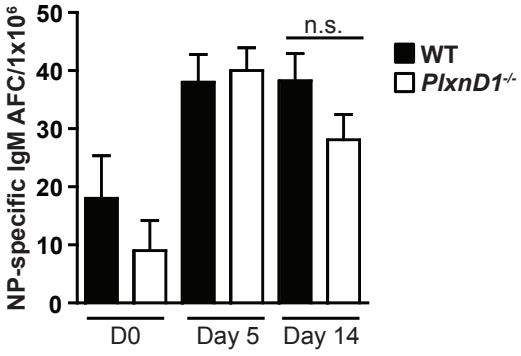

NP-specific IgM AFC/1x10⁶
50
40
30
20
10
0
n.s.
WT
PlxnD1-/-
D0
Day 5
Day 14

Figure 2.12. Normal IgM AFC responses in *PlxnD1*$^{-/-}$ mice.

Antigen specific AFC formation in WT (solid bar) and *PlxnD1*$^{-/-}$ (open bar) mice. Spleens were harvested at day 5 and 14 post-immunization. NP-specific and total IgM AFCs were enumerated by ELISPOT on wells coated with NP_{25}-BSA. Data were pooled from three independent experiments. Mean±SEM. n=10.

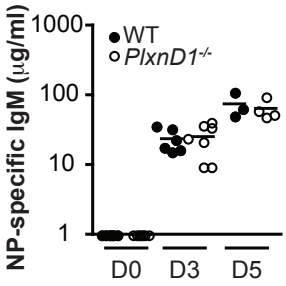

NP-specific IgM (μg/ml)
1000
100
10
1
WT
PlxnD1-/-
D0
D3
D5

Figure 2.13. Normal T_i immune responses in *PlxnD1*$^{-/-}$ mice.

Mice were immunized (ip) with 25 μg NP-Ficoll in PBS. Serum was harvested prior to immunization, 3 and 5 days post immunization. Ab levels were measured by ELISA using NP_{25}-BSA as capture antigens. WT (solid circle) and *PlxnD1*$^{-/-}$ (open circle). NP-specific Ab was detected using goat anti-mouse IgM Ab. Data are representative of two independent experiments. Small horizontal bars indicate mean values. Each circle is representative of one individual mouse.

CHAPTER III

PLEXIN-D1-MEDIATED FUNCTIONS IN THYMOCYTES, LYMPHOCYTE AND DENDRITIC CELLS

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3.1 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-D1 in the immune system. We show that plexin-D1 is highly expressed in DP thymocytes and dendritic cells (DCs). Absence of Plexin-D1 resulted in aberrant thymic development and impaired DP thymocyte migration from the cortex to the medulla. However, the absence of plexin-D1 did not affect mature T cell development and peripheral T cell responses to CFA-KLH challenge. We did not find a role for plexin-D1 in autoimmune disease development as assessed by increased T cell activation and B cell responses. *PlxnD1*$^{-/-}$ DCs and wild-type DCs upregulated costimulatory molecules and activated antigen specific T cells in an indistinguishable fashion. These data indicate that Plexin-D1 is important in early thymic development but does not affect the measured peripheral T cell and DC responses.

3.2 INTRODUCTION

Semaphorins and plexins were initially identified as key molecules in axon guidance during neuronal development (Kolodkin, Matthes et al. 1993; Winberg, Noordermeer et al. 1998). Semaphorins are classified into three different groups based on their origin and structural homology; invertebrate, vertebrate and viral semaphorins (Mizui, Kumanogoh et al. 2009). Plexin receptors are divided into two large groups, invertebrate and vertebrate, and further subdivided into four different families, A-D (Takamatsu, Okuno et al.). Although plexins are considered receptors for the semaphorin ligands, this view is recently being revised as semaphorins are shown to also mediate signal transduction (Tamagnone, Artigiani et al. 1999; Castellani and Rougon 2002; Kruger, Aurandt et al. 2005; Yazdani and Terman 2006). The interactions between semaphorins and plexins are promiscuous because semaphorins can interact with multiple plexins on multiple cell types and vice versa (Takamatsu, Okuno et al.). Plexins and semaphorins control cell movement and migration and are implicated in vasculature formation as well as organ development (Sakurai, Gavard et al. ; van der Zwaag, Hellemons et al. 2002; Gu, Yoshida et al. 2005; Choi, Duke-Cohan et al. 2008).

Recent work has implicated plexins and semaphorins in the immune system (Granziero, Circosta et al. 2003; Wong, Brickey et al. 2003; Walzer, Galibert et al. 2005; O'Connor, Eun et al. 2008; Suzuki, Kumanogoh et al. 2008; Yamamoto, Suzuki et al. 2008). Several plexins and semaphorins are expressed by naïve and activated immune cells. For example plexin-D1 and semaphorin 3E are expressed in the thymus where their interaction is required for proper thymocyte trafficking (Choi, Duke-Cohan et al. 2008). A number of studies have focused on expression of plexins and semaphorins on subsets of DCs and T

cells. For example, plexin-A1 and semaphorin6D are respectively expressed on DCs and activated T cells (Wong, Brickey et al. 2003; O'Connor, Eun et al. 2008) and they regulate different aspects of the DC-T cell interaction during the course of T cell activation. Expression of a number of plexins and semaphorins is associated with an activated cell phenotype. Semaphorin4A is expressed by Th1 polarized T cells and DCs (Kumanogoh, Marukawa et al. 2002); semaphorin4D is expressed by T cells, DCs, and activated B cells (Delaire, Elhabazi et al. 1998; Kumanogoh, Watanabe et al. 2000; Shi, Kumanogoh et al. 2000; Kumanogoh, Suzuki et al. 2002; Granziero, Circosta et al. 2003; Kumanogoh, Shikina et al. 2005); and plexin-B2 is expressed by germinal center B cells (Yamamoto, Suzuki et al. 2008). The studies of semaphorin and plexins expression in the immune system extend to a number of other cell types and are carefully reviewed in (Mizui, Kumanogoh et al. 2009). These studies suggest that immune plexin and semaphorin expression is spatiotemporally regulated as they are expressed by a large number of immune cells at different activation states. As a result, a high degree of cooperation between plexins and semaphorins is expected, as is some redundancy.

Given the high level of plexin and semaphorin expression by a variety of immune cells, a number of studies have been performed in order to address the physiological relevance of these molecules. Work in our laboratory has defined a role for plexin-A1 expression on DCs. This molecule is required for proper T-cell activation and proliferation (Wong, Brickey et al. 2003; Eun, O'Connor et al. 2006). Semaphorin6D, a known ligand for plexin-A1, is expressed on activated T cells and is required for late-phase T cell proliferation (O'Connor, Eun et al. 2008). The role of plexin molecules in T cell immune responses extends to Plexin-A4 (Yamamoto, Suzuki et al. 2008). Mice deficient in plexin-A4 develop

exacerbated MOG-induced experimental autoimmune encephalomyelitis (EAE) (Yamamoto, Suzuki et al. 2008). Semaphorin7A binds to $\alpha 1\beta 1$ integrin and promotes T-cell-mediated immune responses such as contact hypersensitivity (Czopik, Bynoe et al. 2006; Kang, Lee et al. 2007; Suzuki, Okuno et al. 2007). Semaphorin4A is required to promote Th1 immune responses (Kumanogoh, Marukawa et al. 2002). Semaphorin4D maintains B-cell homeostasis and facilitates humoral immune responses(Kumanogoh, Watanabe et al. 2000; Shi, Kumanogoh et al. 2000; Kumanogoh, Suzuki et al. 2002; Kumanogoh, Shikina et al. 2005). Together, these studies suggest that plexin and semaphorins are indispensable for initiation and maintenance of a number of immune responses.

Another member of the plexin family of molecules, plexin-D1, was recently shown to be expressed by DP thymocytes and facilitate their migration from the cortex into the medulla (Choi, Duke-Cohan et al. 2008). However, the role of plexin-D1 in peripheral T cell development and function remains unexplored. To date, plexin-D1 research in the immune system has been limited. Future studies addressing the role of plexin-D1 in T cell immunity would help our understanding of plexin-D1-mediated immune cell migration and activation. Plexin-D1 partners with two different semaphorin molecules: semaphorin3E and semaphorin4A in non-immune cells (Gu, Yoshida et al. 2005; Toyofuku, Yabuki et al. 2007). However, semaphorin3E is the only plexin-D1 partner identified in the immune system (Choi, Duke-Cohan et al. 2008).

Successful T cell development and selection in the thymus is critical for both cellular and humoral immunity. Thymocytes undergo a series of developmental stages where they interact with thymic epithelial cells and DCs in order to be negatively and positively selected (Anderson and Jenkinson 2001). Selection requires controlled movement of thymocytes from

the cortex into the medulla (Benoist and Mathis 1999). Defective migration of thymocytes into the medulla can lead to improper thymocyte selection and subsequently result in impaired T cell immune responses or development of autoimmune disease (Forster, Schubel et al. 1999; Misslitz, Pabst et al. 2004; Ueno, Saito et al. 2004).

To address the role of plexin-D1 in T cell and DC development and function we utilized *PlxnD1*$^{-/-}$ animals. In this study, we report that plexin-D1 is highly expressed by DP thymocytes but not by peripheral naïve and activated T cells. Amond DCs, plexin-D1 is highly expressed by conventional DCs but not plasmacytoid DCs, and its expression is further induced post DC activation. We used *in vitro* and *in vivo* approaches to examine the direct effect of plexin-D1 in T cell and DC immune responses. We found that *PlxnD1*$^{-/-}$ T cells proliferate and produce Th1 and Th2 cytokines similarly to WT T cells, both, *in vitro* and *in vivo*. DCs lacking plexin-D1 were capable of processing antigen and efficiently presenting it to T cells. Together these findings show that plexin-D1 is important for thymocyte migration in the thymus but it does not affect peripheral T cell development and function that were assayed. Additionally, plexin-D1 is dispensable for DC function. These experiments suggest that plexin-D1 might serve redundant functions in mature T cells and DCs, functions that are shared with other plexin molecules.

3.3 RESULTS

Plexin-D1 and semaphorin3E expression in the immune system

A previous study showed that *PlxnD1* is expressed by DP thymocytes (Choi, Duke-Cohan et al. 2008). We extended plexin-D1 expression studies from immature thymocytes to mature peripheral T cells using quantitative real-time PCR methods. *PlxnD1* mRNA expression is minimally detected in peripheral CD4 and CD8 T cells and substantially reduced compared to the thymus and DP T cell progenitors (**Figure 3.1A**). To determine whether activation events affect plexin-D1 expression on T cells, we stimulated sorted $CD4^+$ T cells with anti-CD3 and anti-CD28 (Th0), anti-IL4 and IL12 (Th1) or anti-IFNγ and IL4 (Th2) skewing conditions for 72 hours. Additionally, we stimulated sorted $CD8^+$ T cells with anti-CD3 and anti-CD28 for 72 hours to obtain activated cells. Th0, Th1, and Th2 as well as activated CD8 T cells all expressed little plexin-D1 (**Figure 3.1A**). These data suggest that plexin-D1 expression is confined to immature T cell populations and is not affected by T cell activation events.

We extended plexin-D1 expression studies to another immune cell type, DCs, which are required for T cell development and selection in the thymus and T cell priming in the secondary lymphoid organs. We observed plexin-D1 expression in sorted splenic myeloid DCs (mDCs) ($CD11b^+CD11c^+$) and bone marrow-derived DCs, but not in plasmacytoid DCs ($CD11c^+B220^+mPDCA1^+$) (**Figure 3.1B**). In addition, we activated DCs with various TLR ligands and anti-CD40 Ab and assessed plexin-D1 expression. Our data shows that plexin-D1 is expressed in resting DCs of both spleen and BM origin and further upregulated post TLR ligand stimulation.

Given the high expression of plexin-D1 in the thymus by RT-PCR, we assessed *PlxnD1* expression in the intact thymus of naïve C57BL/6 mice by *in situ* hybridization to confirm plexin-D1 transcript localization. As seen in **Figure 3.1C**, the predominant *PlxnD1* mRNA staining was localized to the cortex and the cortex/medulla border in the thymus. These findings are in line with reports of another group (Choi, Duke-Cohan et al. 2008).

Other studies have shown that the predominant plexin-D1 partner in the immune system is semaphorin3E (Choi, Duke-Cohan et al. 2008). However, these studies do not analyze semaphorin3E expression on specific immune cell populations. We analyzed semaphorin3E expression in a number of immune cells (**Figure 3.1D**). Our data show that semaphorin3E is minimally detected in naïve and activated T and B cell populations. However, semaphorin3E is highly expressed on Th2 skewed T cells and splenic mDCs. The expression pattern of semaphorin3E suggests that partnering of this molecule with plexin-D1 during the course of an immune response may be important for T cell activation and potentially for skewing to a Th2 effector response.

PlxnD1$^{-/-}$ mice exhibit compromised thymic architecture

Given that plexin-D1 is highly expressed in the thymus, we assessed the effect that absence of plexin-D1 has on thymic development. *PlxnD1*$^{-/-}$ animals die shortly after birth thereby preventing the study of intact thymus development later than E19 (Gu, Yoshida et al. 2005). We isolated fetal thymii from embryonic day 19 (E19) animals and observed a reduced size of *PlxnD1*$^{-/-}$ thymii compared to WT mice (**Figure 3.2A**). *PlxnD1*$^{-/-}$ thymii also differed in the shape from WT thymii as they do not appear round.

To further assess whether the size and shape of plexin-D1-deficient thymii affected lymphocyte development in these mice, we performed fetal thymic organ cultures (FTOC). We isolated fetal thymii from E14 animals and cultured them for 7 days before assessing the percentages of thymocytes and T cells present. *PlxnD1*$^{-/-}$ thymii gave rise to similar percentages of single positive CD4 and CD8 thymocytes (**Figure 3.2B**). These data suggest that despite the reduced size and abnormal shape of *PlxnD1*$^{-/-}$ thymii, single positive T cells are produced.

The FTOC system is an *in vitro* cell culture system, thus, assessment of thymocyte development *in vivo* was imperative. Given that *PlxnD1*$^{-/-}$ mice die shortly after birth we created chimeric *PlxnD1*$^{-/-}$ animals where hematopoietic cells from E14 fetal livers were transferred into lethally irradiated congenic mice. This method allowed us to study plexin-D1-deificent thymocyte development post birth. We isolated thymii from chimeric *PlxnD1*$^{-/-}$ mice 4-6 weeks post fetal liver cell transfer. The thymii were then sectioned and stained with CD4, CD8, and CD69 antibodies to determine distribution of thymocytes in the cortex and medulla. As shown in **Figure 3.2C**, thymii of *PlxnD1*$^{-/-}$ reconstituted mice exhibit an abnormal distribution of activated CD69^{+} thymocytes in the cortex of the thymus. Previous studies have reported that activated thymocytes can induce thymic medulla formation in the thymus (Shores, Van Ewijk et al. 1994). We assessed whether activated thymocytes of PlxnD1-/- mice induce medullary structures in the cortex. As shown in **Figure 3.2D**, the frequency of large medullary structures found in the cortex of *PlxnD1*$^{-/-}$ mice is higher than that found in WT animals. These data suggest a role for plexin-D1 in proper cortex-medulla separation and therefore, potential importance in T cell development.

PlxnD1$^{-/-}$ T cells exhibit normal *in vitro* and *in vivo* activation profiles

To determine if the absence of plexin-D1 affects T lymphocyte development in the periphery, C57BL/6 CD45.1 mice were reconstituted with either WT or *PlxnD1*$^{-/-}$ fetal liver cells. Donor and recipient hematopoietic cell populations were distinguished based on the expression of the CD45.1 (host) or CD45.2 (donor) congenic markers. We analyzed the T cell compartment in *PlxnD1*$^{-/-}$ and WT mice. CD4^{-}CD8^{-} (DN), CD4^{+}CD8^{+} (DP), and CD4^{+} or CD8^{+} (SP) thymocytes and mature CD4 and CD8 T cells in the spleen were equivalent in *PlxnD1*$^{-/-}$ and WT animals (**Figure 3.3A**). The *PlxnD1*$^{-/-}$ and WT T cells had similar levels of CD3, TCR-β and CD28 (data not shown).

We next determined the function of mature T cells. Splenic T-cell proliferation in response to anti-CD3 was examined by ^{3}H thymidine incorporation. *PlxnD1*$^{-/-}$ T cells proliferated to levels comparable to the WT controls (**Figure 3.3B**). Sorted CD4^{+}CD62L^{hi} naïve T cells were cultured under Th1 or Th2 skewing conditions. Activated *PlxnD1*$^{-/-}$ CD4^{+} T cells were able to produce similar levels of IL-2, IFNγ, and IL-4 as WT animals (**Figure 3.3C**). These data suggest that despite impaired thymic migration, once the T cells have egressed from the thymus, *PlxnD1*$^{-/-}$ CD4^{+} T cells can produce comparable levels of Th1 or Th2 cytokines.

We immunized *PlxnD1*$^{-/-}$ and WT mice with the antigen KLH in the presence of CFA as well as control OVA-CFA and isolated splenocytes 8 days post immunization. We performed *in vitro* KLH re-challenge of isolated T cells and assessed T cell proliferation using ^{3}H-Thymidine incorporation. As shown in **Figure 3.4A**, T cells exhibit normal proliferative capabilities as compared to WT controls. We assessed T cell cytokine release post *in vitro* recall responses with KLH. As shown in **Figure 3.4B**, WT and *PlxnD1*$^{-/-}$

animals were able to produce similar percentages of activated T cells as assessed by CD44 expression. $CD44^+$ T released similar levels of IFNγ and IL2 cytokines.

These data suggest that plexin-D1 does not affect peripheral T cell function as assessed by T cell proliferation, costimulatory molecules surface expression and Th1 vs. Th2 cytokine release in response to antigenic simulation.

PlxnD1$^{-/-}$ mice do not display signs of autoimmunity when aged

Based on our thymus organization data and previous work published on migration of *PlxnD1$^{-/-}$* DP thymocytes, we tested the hypothesis that *PlxnD1$^{-/-}$* thymocytes might not undergo proper thymic education and lead to autoimmunity in aged *PlxnD1$^{-/-}$* mice. To this end, we aged *PlxnD1$^{-/-}$* animals for 12-14 months and evaluated whether these mice develop autoimmune diseases.

The development of many spontaneous systemic autoimmune diseases is dependent on chronic activation of T cells (Yagi, Matsumoto et al. 1992; Adachi, Inaba et al. 1998). Upregulation of CD44 and downregulation of CD62L are both indicators of T cell activation and potentially T-cell mediated autoimmunity (Chu, Ernst et al. 1994). We assessed CD44 and CD62L surface expression on *PlxnD1$^{-/-}$* and WT T cells by flow cytometry. Our data show that percentages of effector and memory T cells in the spleen were comparable between *PlxnD1$^{-/-}$* mice and WT mice (**Figure 3.5A, B**). Another marker of autoimmune diseases is the presence of anti-dsDNA autoantibody in the serum (Yagi, Matsumoto et al. 1992). Individual serum samples were obtained from 12-14 month old *PlxnD1$^{-/-}$* and WT animals and were tested for the presence of anti-dsDNA autoantibody by ELISA. We did not detect anti-dsDNA autoantibody in the *PlxnD1$^{-/-}$* and WT animals (data not shown).

Our previous work shows a role for plexin-D1 in B cell development and humoral immunity. MZ B cells are a reservoir of autoreactive B cell clones (Martin and Kearney 2002). If *PlxnD1*$^{-/-}$ mice were prone to autoimmune disorders we would expect an expansion of the MZ B cell repertoire. Here we analyzed the MZ B cell compartment in 12-14 month old *PlxnD1*$^{-/-}$ mice. Surprisingly, we observed an almost complete ablation of the MZ B cell compartment in aged *PlxnD1*$^{-/-}$ mice compared to WT mice (**Figure 3.5C**). This finding extends our previous work where we had observed a reduction in the percentages of MZ B cells in 6-10 week old *PlxnD1*$^{-/-}$ animals compared to WT mice. However, the reduction of MZ B cells is much more dramatic in aged mice. Together these findings suggest that plexin-D1 is required for MZ B cell homeostasis through out the lifespan of the mice. Additionally, the MZ B cell compartment in *PlxnD1*$^{-/-}$ mice would not contribute to development of autoimmune disease.

Taken together, these data suggest that ablation of plexin-D1 is not sufficient to induce autoimmune manifestations regardless of the impaired thymocyte migration phenotype observed in the absence of plexin-D1.

Plexin-D1 is not required for DC development

Given the expression of plexin-D1 on DCs and its further upregulation post stimulation, we studied DC development in the absence of plexin-D1. We assessed DC development in the spleen based on expression of CD11c, CD11b and B220. The percentages and numbers of mDCs found in the spleen were equivalent between WT and *PlxnD1*$^{-/-}$ mice (**Figure 3.6A**). We also determine DC formation *in vitro* from bone marrow progenitors of WT and *PlxnD1*$^{-/-}$ reconstituted mice. In the presence of GMCSF and IL4 *PlxnD1*$^{-/-}$ DCs

develop similarly to WT controls (data not shown). Taken together these data suggest that plexin-D1 is not required for development of DCs.

PlxnD1$^{-/-}$ DCs are capable of responding to stimuli and producing cytokines to similar levels as WT controls.

To further assess development of *PlxnD1$^{-/-}$* DCs, we stimulated BMDCs from both *PlxnD1$^{-/-}$* and WT animals with LPS. We determined expression of co-stimulatory molecules on the surface of DCs. As shown in **Figure 3.6B**, CD40, CD80, CD86 and I-Ab surface levels were equivalent between WT and *PlxnD1$^{-/-}$* mice at the basal level and post activation. Supernatants were collected from the different treatments of DC cultures and the levels of cytokines released were measured by ELISA. We determined the levels of IL6, TNFα, and IL12p40 cytokines in these cultures. As shown in **Figure 3.6C**, the IL-6, TNFα and IL12p40 cytokine levels released by cultured DCs were also comparable between WT and *PlxnD1$^{-/-}$* cells.

Taken together, these data suggest that despite expression of plexin-D1 by the DC population and its upregulation post activation, this molecule is not required for upregulation of costimulatory molecules on the surface of activated DCs and for cytokine production by the DCs.

PlxnD1$^{-/-}$ DCs stimulate T cells similarly to WT

To further define the role of plexin-D1 in DCs, we performed an *in vitro* antigen presentation assay using transgenic T cells specific for ovalbumin (OVA), OTII T cells. First we determined the ability of plexin-D1-deficient DCs to take up OVA-protein. DCs were

culture 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. *PlxnD1*$^{-/-}$ and WT DCs take up antigen equivalently as shown by the level of mean fluorescence intensity of the analyzed DCs (**Figure 3.7A**). To determine the ability of the DCs to stimulate T cells, freshly isolated CFSE-labeled OTII T cells were cultured in the presence of OVA protein-pulsed *PlxnD1*$^{-/-}$ and WT splenic DCs for 3 days. Non OVA-pulsed DCs were used as a negative control. Proliferation of V$\beta5^{+}$ OTII T cells was assessed by flow cytometry. As shown in **Figure 3.7B**, plexin-D1-deficient DCs are capable of stimulating T cells similarly to WT. These data suggest that plexin-D1 is dispensable during *in vitro* activation of T cells by the DCs.

3.4 DISCUSSION

Thymocyte migration and selection events are important in ensuring proper T cell activation in the periphery. Recent studies using *PlxnD1*$^{-/-}$ mice have suggested that the plexin-D1-Semaphorin3E interaction is essential for thymocyte migration and possibly selection. In this study, we characterize the expression of plexin-D1 and semaphorin3E in various immune cell types and further determine the role of plexin-D1 in T cell immune responses.

Plexin-D1 is highly expressed by DP thymocytes and myeloid DCs. While its expression is downregulated with T cell maturation and activation, expression by DCs is increased when these cells are activated and matured in the presence of TLR agonists. Expression studies of a plexin-D1 ligand, semaphorin3E, revealed that semaphorin3E is highly expressed by Th2-type T cells and DCs. Based on differential expression of plexin-D1 in lymphoid organs, we hypothesized that in the thymus, absence of plexin-D1 would lead to abnormal T cell development. In the periphery, plexin-D1 might be required for 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 development and activation during immune responses. We demonstrate that *PlxnD1*$^{-/-}$ thymii fail to develop normally. Absence of plexin-D1 in DP thymocytes leads to formation of medullary like structures in the cortex. Our analysis of *PlxnD1*$^{-/-}$ mice did not reveal a role for plexin-D1 in peripheral T cell development and proliferation. *PlxnD1*$^{-/-}$ is dispensable for Th1 and Th2 cell polarization as assessed by IFNγ and IL4 production.

Proper thymocyte migration from the cortex into the medulla ensures central tolerance (Ueno, Saito et al. 2004). T cells are implicated in a series of autoimmune disorders (Yagi, Matsumoto et al. 1992; Adachi, Inaba et al. 1998). For example, mice lacking the *Ccr7* gene and display impaired thymocyte migration develop symptoms of autoimmune disease (Forster, Schubel et al. 1999; Ueno, Saito et al. 2004). An increase in CD44 lymphocyte expression is considered a marker of autoimmune disease (Estess, DeGrendele et al. 1998). For example, spontaneous autoimmune disease in the $MLR^{lpr/lpr}$ mouse strain is accompanied by an increase in percentages of activated T cells expressing high levels of CD44 (Budd, Schumacher et al. 1991). Absence of plexin-D1 does not result in increased percentages of $CD44^+$ T cells in young or aged mice.

A number of other plexin and semaphorin molecules are shown to control development of EAE (Czopik, Bynoe et al. 2006; Yamamoto, Suzuki et al. 2008). Future autoimmune disease models, such as EAE, would be important in establishing a role for plexin-D1 in autoimmune disease development. T cells are key mediators of EAE. It is possible that improper thymic development of $PlxnD1^{-/-}$ T cells could results in accelerated EAE.

Many autoimmune diseases are attributed to B cell hyperproliferation and hyperactivation (Wither, Roy et al. 2000; Segundo, Rodriguez et al. 2001; Groom, Kalled et al. 2002). We have recently shown that plexin-D1 is important for establishment of the GC reaction and subsequently controls recall humoral responses. Impaired T cell selection can lead to aberrant B cell responses and subsequently to development of autoimmune disease (Budd, Schumacher et al. 1991). We did not observe anti-dsDNA antibodies, a hallmark of autoimmune disorders, in the serum of $PlxnD1^{-/-}$ mice. MZ B cells are rapid responders to

antigen challenge and have been implicated in several autoimmune disorders (Martin and Kearney 2002). MZ B cell are enriched with self-reactive clones in autoimmune mice (Wellmann, Werner et al. 2001). Indeed, the MZ B cell compartment expands in NZB/NZW lupus-prone mice prior to disease onset. Interestingly, assessment of the B cell repertoire revealed an almost complete ablation of MZ B cells in aged *PlxnD1*$^{-/-}$ mice. These data point to a role for plexin-D1 in homeostasis of MZ B cells. Plexin-D1 may be needed to provide survival signals to the MZ B cells or it could be required for their ability to adhere to splenic stroma. Taken together, these data show that *PlxnD1*$^{-/-}$ mice do not develop spontaneous autoimmune disorders. In fact, these mice show a reduced MZ B cell compartment. These findings suggest a role for plexinD1 in regulation of immune responses in aging mice. This work could have further implications on targeting of plexinD1 in older individuals in order to promote robust immune responses.

Our studies showed that plexin-D1 is expressed by DCs and further upregulated post DC activation. The T cell-DC interaction is important for T cell selection in the thymus and initiation of immune responses in the periphery. We assessed the role of plexin-D1 in DC development, activation and function. Our results show that plexin-D1 is not required for initial DC development and *in vitro* T cell activation. A number of different plexin molecules control DC responses (Wong, Brickey et al. 2003; Eun, O'Connor et al. 2006) and some degree of cooperation and functional redundancy is expected amongst plexins. Future studies of plexin-D1 deficient DCs are needed in order to define the importance of this molecule in DC biology.

3.5 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 in (Gu, Yoshida et al. 2005). OT-II mice (B6.Cg.Tg(TcrαTCRβ)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*$^{+/-}$ mice were crossed for over 10 generations with C57BL/6 mice and intercrossed to obtain *PlxnD1*$^{-/-}$ and *PlxnD1*$^{+/+}$ embryos. *PlxnD1*$^{+/+}$ and *PlxnD1*$^{-/-}$ fetal livers were prepared from E14 embryos post PCR genotyping as previously described (Gu, Yoshida et al. 2005). Fetal liver cells were then injected (iv) into lethally irradiated C57BL/6 CD45.1 mice. Mice were analyzed 6-10 weeks post reconstitution.

ELISA

Autoantibodies: Sera was obtained from 12-14 month old naïve mice and was tested for anti-dsDNA autoantibodies (IgG, IgM, and IgA) by ELISA (Alpha Diagnostic, San Antonio, TX). Standards, negative, and positive control samples were provided in the kit and were run concurrently according to manufacturer's protocol. *DC cytokine release*: DCs were obtained from *PlxnD1*$^{-/-}$ and WT animals and stimulated for 24 hours in the presence of 1μg/ml anti-CD40 or 50ng/ml LPS. The culture supernatants were tested for IL6, TNFα, and IL12p40 cytokine levels by ELISA (Ebioscience, San Diego, CA).

In Situ Hybridization

Naïve C57BL/6 animals were perfused with PBS and thymii were collected and embedded in OCT compound, snap frozen, and stored at -80°C. The thymii were then cut and fixed in 4%PFA at room temperature for 15 minutes and processed for in situ hybridization by the UNC neuroscience center and in-situ hybridization core. To detect the *PlxnD1* transcript, we used a digoxygenin labeled probe followed by an anti-digoxygenin alkaline phosphotase labeled detection antibody. Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP) was used to visualize the *PlxnD1* transcript.

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), IFNγ (XMG1.2), 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 Alexa350-conjugated streptavidin from Invitrogen (Raleigh, NC). 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 or LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting was performed on a MoFlo cytometer (Beckman-Coulter).

RT-PCR and Quantitative RT-PCR analysis

RNA was isolated from tissues or sorted resting 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'; *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

Proliferation assays: Sorted $CD62L^{hi}$ $CD4^{+}$ and $CD62L^{hi}$ $CD4^{+}$ naive splenic T cells ($2x10^{6}$ cells/ml) were cultured for 3 days with immobilized anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml) in RPMI media (Gibco) containing 10% FBS, 10^{-4}M 2-ME and penicillin/streptomycin (P/S) antibiotics in 96 well plates. One μCi of ^{3}H thymidine was added to the cultures for the last 8 hours to assess proliferation. *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 (5μg/ml) and anti-CD28 (5μg/ml) in the presence of anti-IL4 (10μg/ml) and IL-12 (10ng/ml) (Th1-skewing conditions) or in the presence of anti-IFNγ (10μg/ml) and IL-4 (10ng/ml) (Th2-skewing conditions). Cells were then assessed for cytokine production using intracellular staining and flow cytometry. DC culture: Murine bone marrow DCs were isolated from WT and *PlxnD1*$^{-/-}$

mice and were cultured in the presence of GM-CSF and IL-4 as previously described (van Deventer, Serody 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 50μg/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

Thymii from naïve and immunized 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 TCRβ, CD4, CD8 and CD69 mAb. FITC signal was amplified using anti-FITC-Alexa488 mAb. Streptavidin-AlexaFluor350 or Streptavidin-AlexaFluor405 was used to amplify -biotin signal (blue). Images were acquired using a Zeiss Axiovert 200M or Zeiss LSM 710 confocal immunofluorescent microscope.

Statistical analysis

Statistical significance was determined with two-tailed Student's t test. All p values less than 0.05 were considered significant.

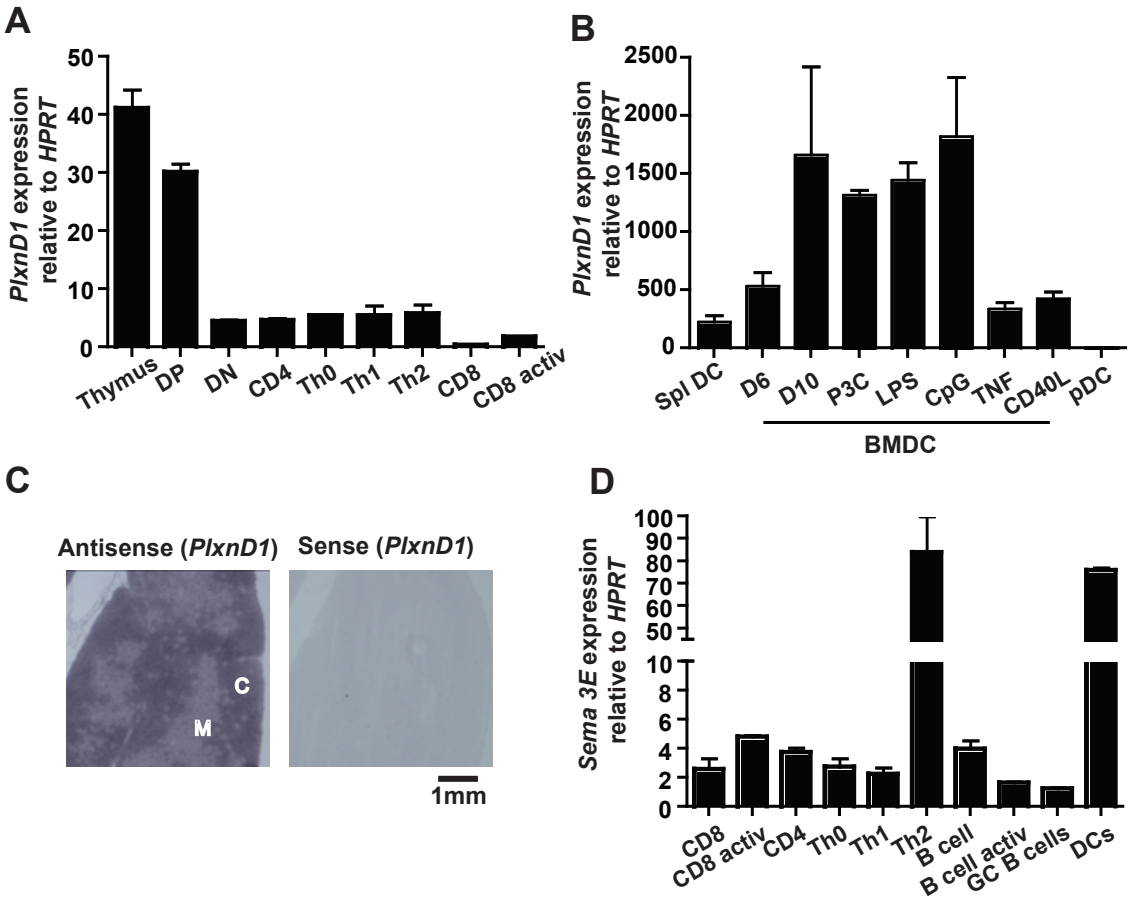

A
PlxnD1 expression relative to HPRT
50
40
30
20
10
0
Thymus
DP
DN
CD4
Th0
Th1
Th2
CD8
CD8 activ
B
PlxnD1 expression relative to HPRT
2500
2000
1500
1000
500
0
Spl DC
D6
D10
P3C
LPS
CpG
TNF
CD40L
pDC
BMDC
C
Antisense (PlxnD1)
Sense (PlxnD1)
C
M
1mm
D
Sema 3E expression relative to HPRT
100
90
80
70
60
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. Plexin-D1 expression. (A) Expression of *PlxnD1* in the thymus, DN and DP thymocytes, SP thymocytes and activated CD4 and CD8 T cells as measured by real-time PCR. Data are representative of three independent experiments. **(B)** Expression of *PlxnD1* in sDCs, BMDC D6 and D10, and activated BMDC as measured by real-time PCR. Data are representative of three independent experiments. **(C)** Expression of *PlxnD1* as measured by *in situ* hybridization. *In situ* hybridization was performed using a digoxygenin labeled probe recognizing *PlxnD1* mRNA (bp 1750-2468) on 6-8 week old C57BL/6 thymus sections. Data representative of 2 independent experiments. **(D)** Expression of Semaphorin3E in sorted naïve and activated T cell and B cell populations, and DCs. Data are representative of 3 independent experiments.

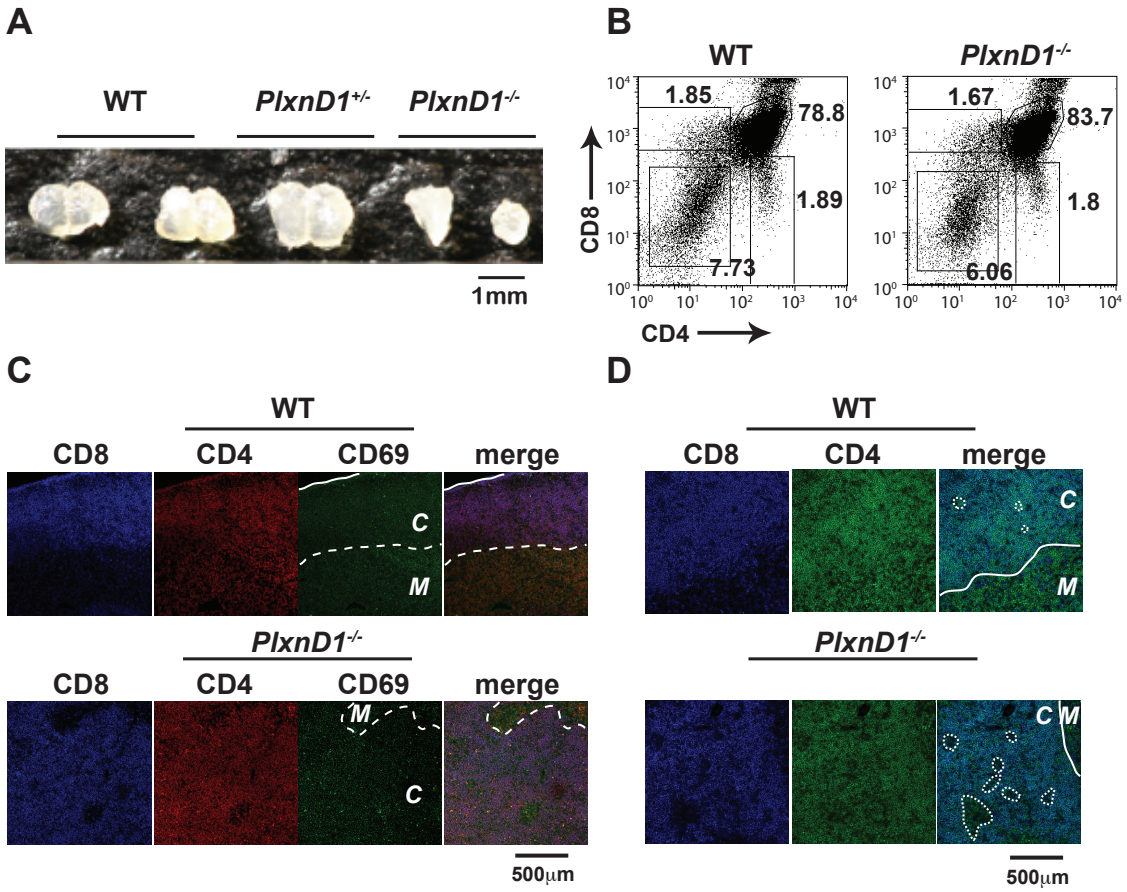
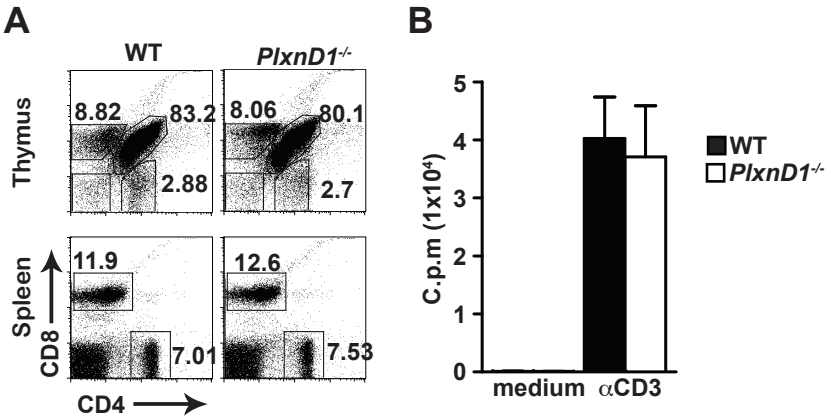

A
WT
PlxnD1+/-
PlxnD1-/-
1mm
B
WT
PlxnD1-/-
1.85
78.8
1.89
7.73
1.67
83.7
1.8
6.06
CD8
CD4
C
WT
CD8
CD4
CD69
merge
C
M
PlxnD1-/-
CD8
CD4
CD69
merge
M
C
500μm
D
WT
CD8
CD4
merge
C
M
PlxnD1-/-
C
M
500μm

Figure 3.2. Abnormal thymic architecture and medullary structure formation in the absence of plexin-D1. (A) Thymii were collected from E19 embryos and images were acquired using a digital camera. Images are representative of 5 different experiments. n=8-10 mice per group. **(B)** Thymii were collected from E14 embryos and cultured for 3 days. Single cells suspensions were prepared and analyzed by flow cytometry using anti-CD4 and -CD8 mAbs. **(C)** Five mm sections of thymii from WT and *PlxnD1*$^{-/-}$ chimeric mice were labeled with CD8-AF405 (blue), CD4-PE (red) and CD69-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. n=10 mice per group. **(C)** Five mm sections of thymii from WT and *PlxnD1*$^{-/-}$ chimeric mice were labeled with CD8-AF405 (blue) and CD69-FITC (green) antibodies. FITC signal was amplified using anti-FITC-AF488 Ab. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. n=10 mice per group.



C

	Th0			Th1			Th2		
	IL2	IFNγ	IL4	IL2	IFNγ	IL4	IL2	IFNγ	IL4
WT (% of total)	10.4 ±2.1	5.9 ±1	0.35 ±0.05	22.5 ±2.3	21.5 ±1.5	0.2 ±0.01	6.6 ±1.3	6.2 ±0.9	9 ±0.7
***PlxnD1*$^{-/-}$** (% of total)	11.2 ±2.4	5.8 ±0.1	0.55 ±0.03	25.1 ±3.4	18 ±1	0.19 ±0.02	6.4 ±0.9	6.9 ±2.2	8.05 ±0.4

Figure 3.3. Normal reconstitution and *in vitro* function of *PlxnD1*$^{-/-}$ T cells

(A) Thymus and spleen cells were harvested from *PlxnD1*$^{-/-}$ and WT mice and analyzed by flow cytometry. Monoclonal Ab to CD45.1, CD45.2, CD4, and CD8 were used to stain different T cell progenitors and mature T cells in the thymus **(top)** and spleen **(bottom)**. Data are representative of 3 different experiments. n=12 mice per group. **(B)** $CD62L^{hi}CD4^{+}$ T cells were purified and cultured for 72 hours with anti-CD3. Cells were pulsed with ^{3}H thymidine for the last 8 hours in culture to determine cell proliferation and data are presented as counts per minute (c.p.m). Data are representative of 3 different experiments. n=9 mice per group. **(C)** $CD62L^{hi}CD4^{+}$ T cells were purified and cultured for 72 hours with anti-CD3/anti-CD28 in the presence of Th1 or Th2 skewing conditions. IL2, IFNγ and IL4 cytokine production were assessed by intracellular staining. Data are representative of 3 different experiments. n=9 mice per group.

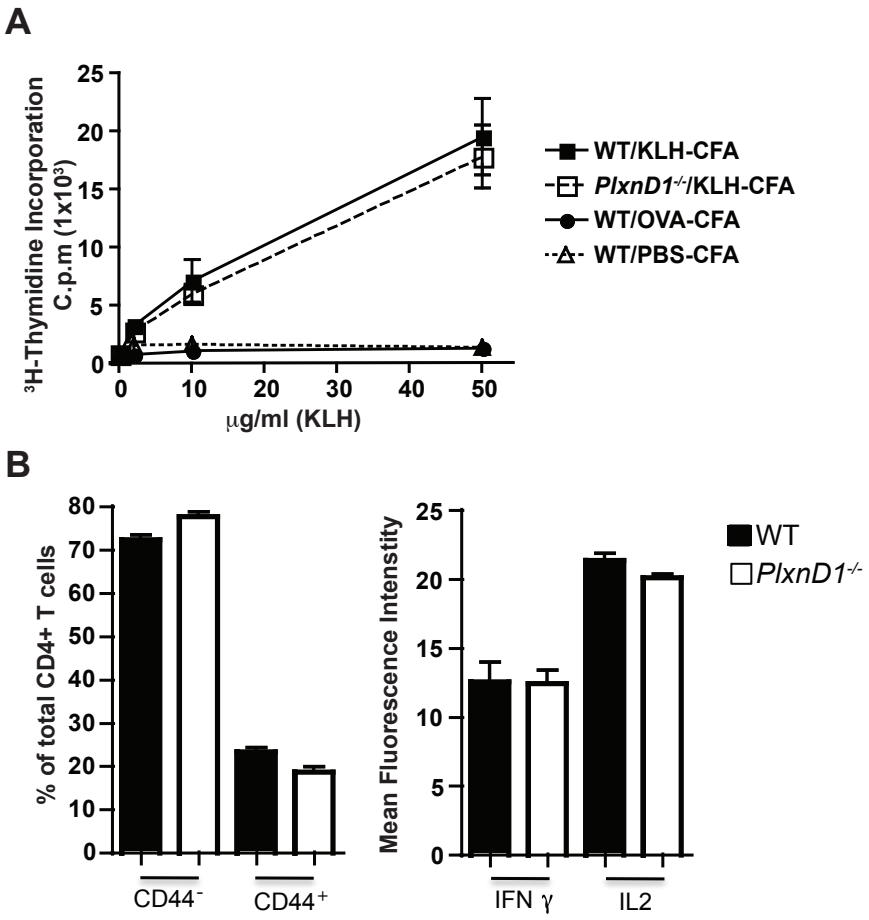

A
^{3}H-Thymidine Incorporation C.p.m (1x10^3)
μg/ml (KLH)
WT/KLH-CFA
PlxnD1$^{-/-}$/KLH-CFA
WT/OVA-CFA
WT/PBS-CFA
B
% of total CD4+ T cells
CD44$^-$
CD44$^+$
Mean Fluorescence Intenstity
IFN γ
IL2
WT
PlxnD1$^{-/-}$

Figure 3.4. Normal recall responses of *PlxnD1*$^{-/-}$ T cells. (A) Chimeric WT and *PlxnD1*$^{-/-}$ mice were immunized with KLH-CFA, OVA-CFA, or PBS-CFA and analyzed 7 days post immunization. Splenic T cells were isolated and cultured in the presence of DCs and varying amounts of KLH for 3 days. CD4 T cell proliferation was assessed via ^{3}H-thymidine incorporation. **(B)** Splenocytes from KLH-CFA immunized mice were cultured in the presence of KLH for 6 hours and cytokine production was analyzed by flow cytometry. Data are representative of 3 independent experiments. n=8 mice per group.

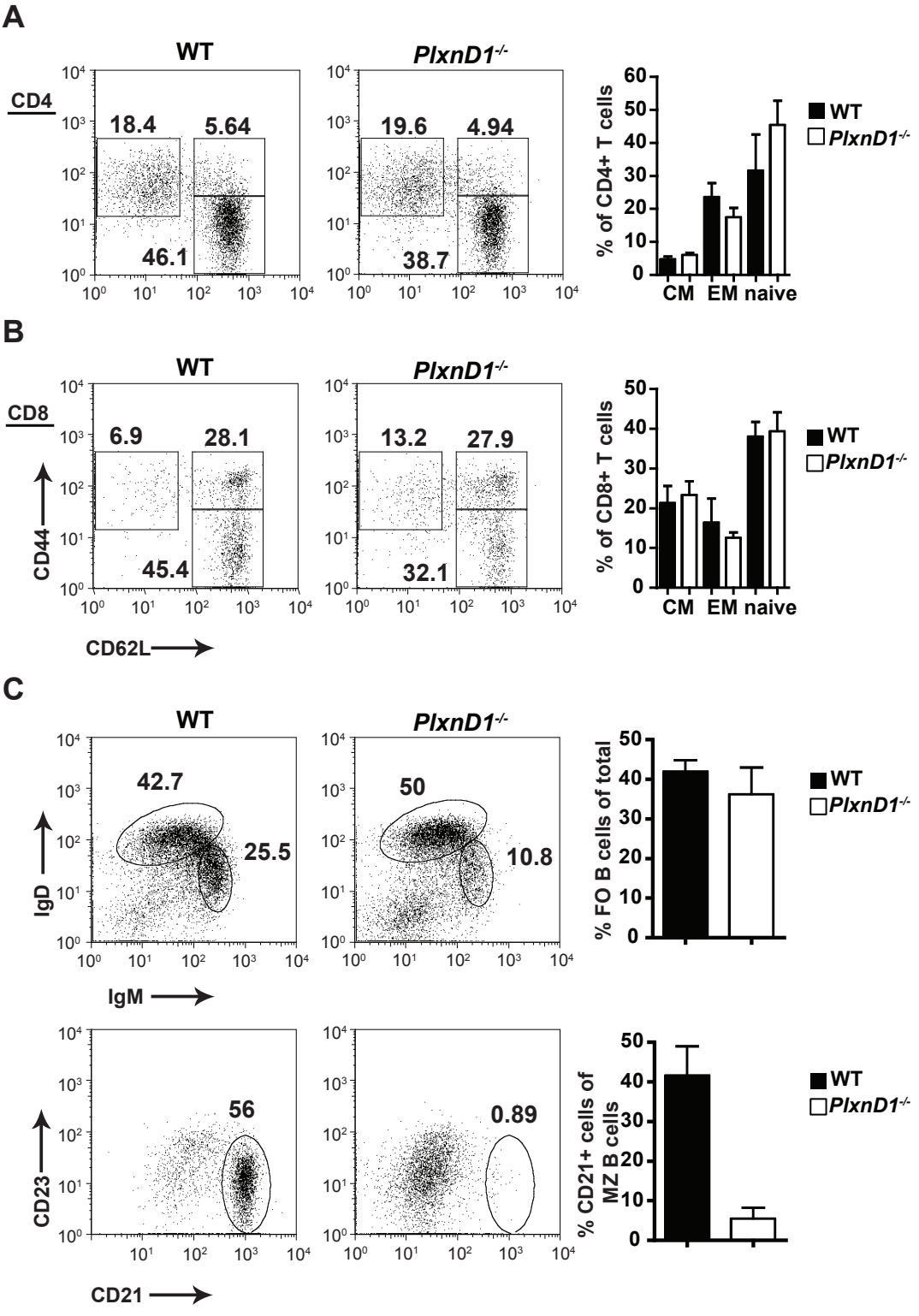

A
WT
PlxnD1-/-
CD4
18.4
5.64
46.1
19.6
4.94
38.7
% of CD4+ T cells
CM EM naive
WT
PlxnD1-/-
B
WT
PlxnD1-/-
CD8
6.9
28.1
45.4
13.2
27.9
32.1
CD44
CD62L
% of CD8+ T cells
CM EM naive
WT
PlxnD1-/-
C
WT
PlxnD1-/-
42.7
25.5
50
10.8
IgD
IgM
% FO B cells of total
WT
PlxnD1-/-
56
0.89
CD23
CD21
% CD21+ cells of MZ B cells
WT
PlxnD1-/-

Figure 3.5. Aged *PlxnD1*$^{-/-}$ animals do not show signs of autoimmunity. Chimeric WT and *PlxnD1*$^{-/-}$ mice were aged for 12-14 months. Splenocytes were isolated and CD4 **(A)** and CD8 **(B)** effector and memory cells were analyzed by flow cytometry. Data are representative of 2 different experiments. N=6 mice per group. **(C)** Splenocytes were isolated and stained with antibodies against B220, CD21, CD23, IgM, and IgD. Cells were anaysed by flow cytometry. Data are representative of 2 different experiments. n=6 mice per group.

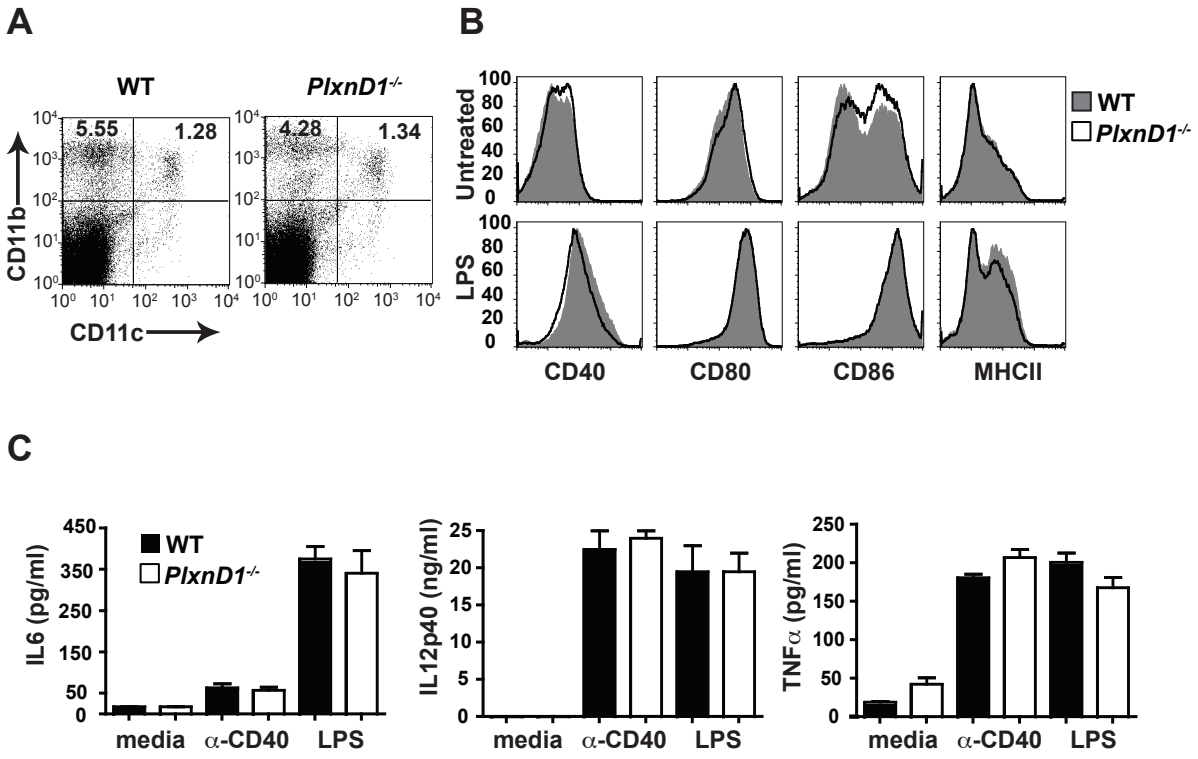

A
WT
PlxnD1-/-
5.55
1.28
4.28
1.34
CD11b
CD11c
B
Untreated
LPS
WT
PlxnD1-/-
CD40
CD80
CD86
MHCII
C
IL6 (pg/ml)
WT
PlxnD1-/-
media
α-CD40
LPS
IL12p40 (ng/ml)
TNFα (pg/ml)

Figure 3.6. *PlxnD1*$^{-/-}$ DCs develop and function similarly to WT controls.

(A) Spleens were harvested from *PlxnD1*$^{-/-}$ and WT mice and splenocytes were analyzed by flow cytometry. Monoclonal Ab to CD45.1, CD45.2, CD11b and CD11c were used to stain DCs in the spleen. Data are representative of 3 different experiments. n=9 mice per group. **(B)** *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 *PlxnD1*$^{-/-}$ and WT 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=9 mice per group. **(C)** *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=9 mice per group.

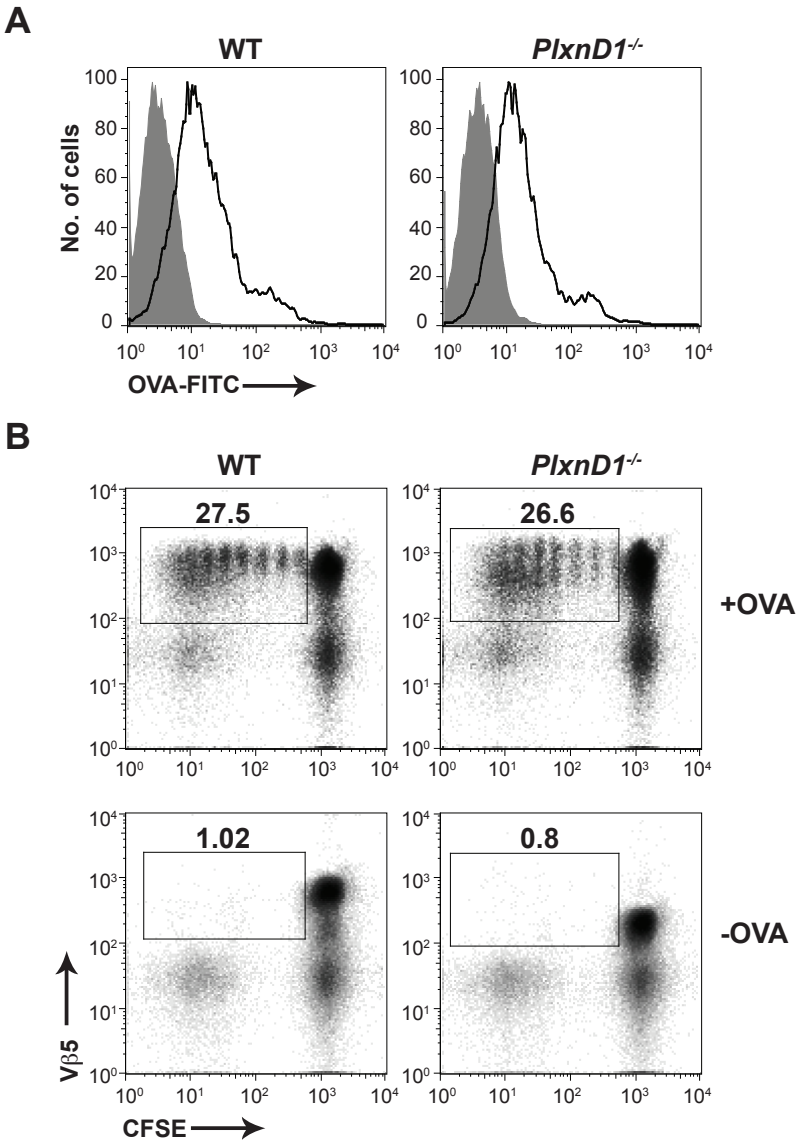

A
WT
PlxnD1-/-
No. of cells
OVA-FITC
B
WT
PlxnD1-/-
27.5
26.6
+OVA
1.02
0.8
-OVA
Vβ5
CFSE

Figure 3.7. *PlxnD1*$^{-/-}$ DCs efficiently stimulate antigen specific T cells. *PlxnD1*$^{-/-}$ DCs are capable of stimulating T cells and inducing proliferation. **(A)** OVA uptake for *PlxnD1*$^{-/-}$ and WT DCs. DCs were isolated from spleens of mice reconstituted with *PlxnD1*$^{-/-}$ and WT 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 *PlxnD1*$^{-/-}$ and WT 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.

CHAPTER IV

DISCUSSION

The discovery of plexin and semaphorin molecules in the immune system has opened several avenues for future research. A large number of these plexin and semaphorin molecules remain understudied in the context of immune responses. Research has demonstrated that plexins utilize several semaphorin ligands and other receptors on different cell types to regulate cellular interactions (Kruger, Aurandt et al. 2005; O'Connor, Eun et al. 2008; Suzuki, Kumanogoh et al. 2008). The aim of this dissertation is to elucidate the role of plexin-D1 in the immune system. Plexin-D1 was chosen for its high expression on immune cells, specifically DP thymocytes, B cells and DCs. We examined the role of plexin-D1 during the course of B and T cell immune responses in *PlxnD1*$^{-/-}$ mice.

A major portion of this dissertation demonstrates a role for plexin-D1 in the regulation of humoral immune responses. The humoral arm of the adaptive immune system is necessary to provide Abs that protect the organism from pathogen encounters, both, in the short and long term (Renshaw, Fanslow et al. 1994)).

As described in chapter 2, plexin-D1 was constitutively expressed by MZ B cells and induced upon activation of FO B cells. Given that plexin-D1 expression was associated with an activated B-cell phenotype, we further investigated the importance of plexin-D1 in B cell immunity. Absence of plexin-D1 did not affect B cell development in the BM or spleen with the exception of marginal zone B cell numbers. As a result, we investigated the ability of plexin-D1 deficient B cells to produce antibody. At basal level, *PlxnD1*$^{-/-}$ mice showed reduced levels of class-switched IgG1 and igG2 antibodies. Further analysis revealed that mice lacking plexin-D1 have reduced GC formation and maintenance. This in turn led to impaired antibody recall-responses. To determine if the B-cell phenotype could be rescued by the presence of plexin-D1 sufficient B cells we created chimeric animals where 50% of

donor cells were of wild-type origin. *In vivo*, *PlxnD1*$^{-/-}$ B cells did not migrate into the GC as efficiently as WT B cells suggesting that the GC phenotype we observe was B cell specific and could not be rescued in the presence of WT T cells. As an analogy to our *in vivo* experiments, we performed *in vitro* B-cell migration assays. *PlxnD1*$^{-/-}$ B cells showed reduced migration towards GC chemokines; CCL19, CXCL12, and CXCL13. Thus, we correlate the impaired GC reaction in *PlxnD1*$^{-/-}$ mice to the inability of plexin-D1 deficient B cells to migrate towards GC chemokines.

New research avenues and questions arise as a result of these findings. What are the patterns of *PlxnD1*$^{-/-}$ B-cell movement during a T_d immune response and the GC reaction? What cellular events, in addition to migration, contribute to impaired GC formation? Is the pool of memory B cells directly affected by the reduced GC reaction? Are there other molecules that interact with plexin-D1 and control T_d humoral immune responses, specifically the GC reaction? What other molecules does plexin-D1 interact with during the T_d humoral immune response, specifically the GC reaction?

What are the patterns of *PlxnD1*$^{-/-}$ B-cell movement during a T_d immune response and the GC reaction?

In chapter 2 we show that plexin-D1-deficient B cells display an impaired ability to migrate into the GC. Future work studying the movement of *PlxnD1*$^{-/-}$ B cells during the course of a T_d immune response is needed. An extensive study employing two-photon/multi-photon microcopy to track plexin-D1 deficient B cells could be used to determine whether cells enter, but are not retained in, the GC reaction. Using 2-photon or multiphoton microcopy, we could track plexin-D1-deficient B cells within the GC and determine

whethere *PlxnD1*$^{-/-}$ B cells *i*) are able to collaborate with Th cells found in the GC, *ii*) can move between the LZ and DZ, and *iii*) are able to be retained in the GC.

What cellular events, in addition to migration, contribute to impaired GC formation?

In part, we attributed impaired GC formation to the inability of *PlxnD1*$^{-/-}$ B cells to migrate into the GC as efficiently as WT B cells This was followed by reduced migration of *PlxnD1*$^{-/-}$ B cells towards GC chemokines. We correlate impaired migration into GC reactions with reduced in vitro migration of *PlxnD1*$^{-/-}$ B cells to chemokines present in GCs. However, the *in vitro* migration defects are incomplete, thus suggesting that plexin-D1 might affect other processes required for initiation and maintenance of the GC. B cell proliferation and apoptosis are important aspect of GC formation and maintenance (Linterman, Beaton et al. ; Zotos, Coquet et al.). For example, studies of *IL21*$^{-/-}$ mice show reduced GC B cell proliferation and subsequently impaired GC maintenance (Linterman, Beaton et al. ; Zotos, Coquet et al.). Similar to this work, we would determine proliferation of *PlxnD1*$^{-/-}$ B cells in the GC reaction using BrdU incorporation studies. Although we have not observed differences in B cell proliferation *in vitro*, the GC reaction utilizes a series of cytokines to organize cellular compartments *in vivo*, to drive proliferation. Accordingly, we would expect the proliferative response that occurs during the GC reaction to differ from the proliferative response observed *in vitro* with purified B cells.

Apoptosis is an important aspect of the GC response (MacLennan 1994; Koopman, Keehnen et al. 1997; Allen, Okada et al. 2007). Our data does not address whether Plexin-D1$^{-/-}$ B cells have an increased propensity towards apoptosis. Determining the rate of apoptosis between *PlxnD1*$^{-/-}$ and WT GC B cells would be difficult; however, studies in Bcl-

X_L transgenic *PlxnD1*$^{-/-}$ mice would help determine whether enhanced apoptosis contributes to impaired GC response.

Cell adhesion is required for proper B cell activation and selection in the GC reaction (Koopman, Keehnen et al. 1994; Balogh, Aydar et al. 2002). Examination of adhesions molecule expression patterns on *PlxnD1*$^{-/-}$ GC B cells would provide insight into which components are controlled by plexin-D1. This could be achieved via microarray studies, followed by real-time RT-PCR and western blot studies. Several groups are interested in the role of plexins and the immune system; therefore, we expect a plexin-D1-specific monoclonal-Ab to become available. Availability of this monoclonal-Ab against plexin-D1 will allow biochemical studies to determine interacting partners of plexnD1 following B cell activation.

Plexin molecules act as regulators of Rho family small GTPases to control cell movement and adhesion in response to extracellular stimuli (Zanata, Hovatta et al. 2002; Oinuma, Katoh et al. 2003; Tong, Chugha et al. 2007; Uesugi, Oinuma et al. 2009). Rnd proteins are new members of the Rho family, which lack intrinsic GTPase activities (Nobes, Lauritzen et al. 1998). Instead, Rnd proteins partner with other molecules such as p190-RhoGAP or Pragmin to control Rho family GTPases such as RhoA (Katoh, Harada et al. 2002; Wennerberg, Forget et al. 2003; Tanaka, Katoh et al. 2006). Rnd proteins also bind the cytoplasmic domain of plexin molecules in order to promote semaphoring-plexin signaling. For example, plexin-B1 encodes a GTPase-activating protein (GAP) for R-Ras in the cytoplasmic domains (Oinuma, Ishikawa et al. 2004). Plexin-B1 partners with Rnd1 in order to form a molecular complex that that stimulates the GTPase activity of R-Ras(Oinuma, Katoh et al. 2004). This in turn regulates cell migration. Research on plexin-D1 has revealed

that this molecule displays R-Ras GAP activity in the presence of Rnd2 and control cell movement (Uesugi, Oinuma et al. 2009). The intrinsic R-Ras GAP domains on plexin molecules are likely to be a common signaling component that extends to immune cell types and is used for plexin signaling during the course of an immune response.

Interruption of plexin-D1 expression at different time points during the T_d B cell immune response would help determine whether plexin-D1 is required for initiation or maintenance of the GC. Blocking the R-Ras activity of plexin-D1 during B-cell immune responses could lead to clues as to how plexin-D1 controls B cell migration. This could be accomplished by administration of plexin-D1 blocking agents, such as a plexin-D1-Fc fusion protein, at different time points after antigen immunization.

Is the pool of memory B cell directly affected by the reduced GC reaction?

The GC is the pivotal site of memory B cell formation (McHeyzer-Williams and McHeyzer-Williams 2005). How plexin-D1 affects memory B cell development is still under investigation. Our work shows a requirement for plexin-D1 in antibody recall responses and long-lived BM plasma cell formation. Preliminary studies in our laboratory have shown that plexin-D1 does not affect development of antigen specific B cells with a memory phenotype, as determined by expression of surface CD38. However, the frequency of these cells is reduced in *PlxnD1*$^{-/-}$mice compared to WT. The mechanism for this outcome needs to be explored in the future. This could be achieved by studying the cell signaling events that take place during the GC reaction. For example, GC signaling pathways, such as p53, Bcl-6, and Blimp-1 could be examined via Western Blot analysis and Phospho-Flow. We have not examined whether *PlxnD1*$^{-/-}$ memory B cells are capable of functioning similarly to WT

cells. Future experiments where memory B cells are transferred into naïve recipients and assessed for their ability to expand and respond to antigenic challenge would be particularly informative.

Are there other molecules that interact with plexin-D1 and control T_d humoral immune responses, specifically the GC reaction?

To date, two plexin-D1 binding partners have been identified: Sema3E and Sema4A (Gu, Yoshida et al. 2005; Toyofuku, Yabuki et al. 2007);however, plexins and semaphorins can have multiple interacting partners (Kruger, Aurandt et al. 2005; O'Connor, Eun et al. 2008; Suzuki, Kumanogoh et al. 2008). Thus, it is possible that Plexin-D1 utilizes different semaphorin molecules and/or other ligands at various stages of the immune response. Although we have determined the expression pattern of Sema3E (Figure 3.1), further studies would help establish whether Sema3E is the plexin-D1 partner during B cell immune responses. Plexin-D1 studies in the vascular system have shown that plexin-D1-Sema3E partnering can have repulsive or attractive properties in the absence or presence of neuropilin-1 binding, respectively (Gu, Yoshida et al. 2005; Toyofuku, Yoshida et al. 2008).

We hypothesize that during the course of a T_d immune response, plexin-D1 utilizes different binding partners to coordinate cellular localization. For example, at the initiation of the immune response, plexin-D1 could use Sema3E to facilitate B cells migration towards the T cell zone. Once B cells have received T cell help, surface plexin-D1 could partner with other ligands to force B cell migration back into the B cell follicle and ultimately into the GC reaction. Identifying the specific partners for Plexin-D1 will help shed light on the molecular

mechanisms that contribute to the B cell response and potentially identify future targets for vaccine design.

T cell and DC biology

As described in chapter 3, plexin-D1-deficient double positive thymocytes exhibited impaired migration from the cortex into the medulla. This defect in migration led to compromised thymic architecture; however, the absence of plexin-D1 did not affect peripheral T-cell development. Independently, Choi *et al* identified a role of plexin-D1 in T cell development in the thymus (Choi, Duke-Cohan et al. 2008). This work demonstrated that plexin-D1 was important for thymocyte migration from the cortex into the medulla. These studies did not examine the role of plexin-D1 in T cells past the thymic stages of development. These authors speculated that *PlxnD1*$^{-/-}$ mice might foster autoimmunity with age, based on previous research with *Ccr7*$^{-/-}$ mice (Ueno, Saito et al. 2004). Similarly to *PlxnD1*$^{-/-}$ mice, *Ccr7*$^{-/-}$ mice exhibit abnormal thymic architecture due to impaired migration of DP thymocytes into the medulla. Our results presented in Chapter 3 further extend this work to determine the functionality of T cell in the periphery.

PlxnD1$^{-/-}$ T cells responded similarly to WT T cells when activated both *in vitro* and *in vivo*. Aged *PlxnD1*$^{-/-}$ mice did not develop autoreactivity as assessed by the normal frequency of activated T cells in the spleen, lack of auto-antibodies in the serum, and lack of immune complexes deposition in the kidneys of the mice. Although, *PlxnD1*$^{-/-}$ mice showed no obvious signs of autoreactivity, a more extensive study of other organs such as lachrymal glands could reveal an autoimmune phenotype. Future studies could also focus on T effectors cell development, TCR repertoire and TCR affinity analysis.

Many genes that contribute to the development of autoimmune diseases are not apparent on the C57BL/6 background. For example, *TRAF3*$^{-/-}$ mice develop autoimmunity on a mixed background such as B6 x 129 (Xie, Stunz et al. 2007). Therefore, it would be important to study the effects of plexin-D1 deficiency and its ability to induce autoimmune disease on a different genetic background such as the B6 x 129.

DC-T cell interaction

Our work in chapters 2 and 3 clearly demonstrates the requirement for Plexin-D1 in *i*) thymocyte movement, *ii*) in GC development and, *iii*) humoral recall responses. Thymic T cell selection and the GC reaction are complex processes that require DCs for antigen presentation and selection (Anderson and Jenkinson 2001; Allen and Cyster 2008). Plexin-D1 is highly expressed by DCs and its expression is upregulated with activation. As detailed in chapter 3, *PlxnD1*$^{-/-}$ DCs develop similarly to WT DCs. They are capable of activating T cells and producing an array of cytokines.

Our peripheral T cell and DC studies raise several questions about the role of plexin-D1 in DC-T cell biology. Does absence of plexin-D1 affect T cell memory responses? How do *PlxnD1*$^{-/-}$ mice respond to live viral or bacterial challenge? Are *PlxnD1*$^{-/-}$ mice protected or susceptible to diseases such as EAE? We would expect a role for plexin-D1 in T cell mediated immune responses. However, it is possible that there are other mechanisms in place to compensate for the absence of plexin-D1 in these cells types. Given the large number of plexin molecules and their abundant expression in the immune system, it is possible that some of these molecules have functional redundancy.

Our research to date suggests that plexin-D1 expression is tightly regulated in the immune system. Plexin-D1 regulates migratory patterns of developing thymocytes and activated B cells. Future work would compare regulation of plexin-D1 expression to that of chemokines and their receptors. This would allow us to better understand spatiotemporal regulation of immune cell migration. Chemokines might be important in recruiting immune cells to immune organs and help direct them to specific areas such as B-cell follicles or T cell zones. Plexin molecules may play important roles in fine-tuning these migratory processes and regulating immune cell migration into microenvironments. Additionally, cytokines are soluble molecules with low specificity for cell-migration. Plexins and semaphorins can help regulate cell migration via cell-cell interactions, thus increasing the specificity of immune cell migration in specific zones. Due to its ability to regulate different stages of cell development and immune responses, specifically in the formation of GCs and the generation of Ab-producing cells, plexin-D1 is great candidate for future research towards vaccine design.

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