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THE FUNCTION OF A NOVEL GUIDANCE MOLECULE, PLEXIN-A1, ON IMMUNE DENDRITIC CELLS

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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.

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ABSTRACT

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The Function of a Novel Guidance Molecule, Plexin-A1, on Immune Dendritic Cells

(Under the direction of Jenny P-Y. Ting)

Plexins (Plxns) and their ligand molecules, semaphorins were originally known as axonal guidance factors in neurons. PlxnA1, in particular, was discovered in neurons along with its soluble ligand Sema3A, another neuronal guidance cue. Since then, it has also been implicated in a variety of contexts such as cardiovascular development, carcinogenesis, or immune responses. In the immune system, PlxnA1 was originally detected in bone marrow-derived dendritic cells (DCs), regulated by Class II Transactivator (CIITA), the master regulator of MHC class II molecules. PlxnA1 expression was shown highly upregulated in mature DCs where it appeared to play a crucial role in T cell priming. The work presented here demonstrates a dual role of PlxnA1 on the surface of DCs in cognate T cell priming upon conjugation as well as in chemokine-induced DC migration. The mechanism by which PlxnA1 stimulates T cell activation upon contact involves small GTPases. PlxnA1 stimulates naïve T cell activation in an antigen-dependent manner, and augments chemotaxis of DCs towards defined chemokines attracting mature DCs, such as CCL19/21, and CXCL12, as

demonstrated using DCs lacking PlxnA1. It was observed that upon T cell engagement, PlxnA1-deficient DCs contained significantly reduced levels of Rho-GTPase, leading to a defect in the polarization of actin filaments towards the interface with T cells downstream of Rho. As also suggested by a previous observation that *plxnA1*$^{-/-}$ mice showed reduced symptoms of experimental autoimmune encephalomyelitis upon challenge, PlxnA1 presents its potential to be a target for developing therapeutics against autoimmune disorders among others.

DEDICATION

This work is dedicated to

my extraordinary grandmother, Ms. Soon-Rye Park, who I miss everyday,

and my loving mother as well as my best friend, Dr. Hioh-Il Choe.

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Portions of this dissertation were previously published or are in preparation for submission for publication. I would like to express my appreciation to the co-authors who have contributed to this work.

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CHAPTER I: INTRODUCTION

1.1. Plexins and Semaphorins

1.1.1. Plexins.

Plexins (Plxns) and Semaphorins were initially studied in the neuronal system. The name of Plxn was derived from the plexiform layers of the optic tectum and the neural retina where its expression was primarily observed [1, 2]. The Plxn family is divided into four subfamilies designated A through D, and comprises nine members (Figure 1.1) [3]. In the murine species, the A subfamily of Plxns consists of four members including PlxnA1 which is the main focus of this project [3]. Plxns are well-conserved throughout the animal kingdom from invertebrates to vertebrates, which suggests an important role in normal function and development [3-7].

Plxns are type I transmembrane glycoproteins, and remain membrane-bound except for the B type subfamily in vertebrates that contain convertase-cleavage sites that lie adjacent to the membrane-spanning region [2, 8-10]. Indeed, in human cell lines and tissue samples, cleaved B type Plxns such as PlxnB1 and PlxnB2 are the predominantly found [8]. The shorter fragment cleaved from the full-length protein contains the entire cytoplasmic portion plus the membrane-spanning region along with a very short extracellular part of the full length protein, whereas the longer fragment contains most of the N-terminal extracellular part before the cleavage site. It has been suggested that proteolytic processing of B type Plxns by proprotein convertases occurs in post-Golgi compartments and or at the cell surface. However, according to the observation, the two cleaved fragments (a shorter fragment: ~100kDa; a longer one: ~200kDa) were still weakly bound together as a heterodimer via non-disulfide linkage. Moreover, compared to a noncleavable mutant form of the protein, the heterodimer exhibited higher affinity for their ligand, Sema4D, leading to a stronger collapse

response of fibroblasts [8]. Consequently, the heterodimer of B type Plxns seems to remain plasma membrane-bound and respond to surface ligands. However, another report suggested that a secreted PlxnB1 protein exists in humans. The authors stated that two different versions of PlxnB1 mRNAs were found, one of which was full-length, but the other a splice variant that was truncated within the extracellular portion of the protein, due to a frame shift in the open reading frame [3]. Thus, it is plausible that B type Plxns could be released into the extracellular environment spontaneously or upon ligand stimulation. However, the question of whether and how such events take place is left unknown.

The N-terminal Sema domain of Plxns is responsible for ligand-binding [11-13]. The Sema domains are shared by Plxns, Semaphorins, and the proto-oncogenic MET and RON (Recepteur d'Origine Nantais) receptor tyrosine kinases (RTKs). The Sema domain is known to mediate protein-protein interactions including Semaphorin dimerization as well as Semaphorin interactions with Plxn and Neuropilin (Nrp) [3, 11-15]. The Sema domain is also an autoinhibitory element for Plxn activity, in the resting state. A truncated mutant of Plxn protein lacking the Sema domain was shown to be constitutively active and nonresponsive to ligand stimulation, suggesting that Plxns undergo a conformational change upon ligand-binding [16]. Crystal structures of Sema3A and Sema4D revealed that the Sema domain is the largest known among the seven-blade β-propeller folds, approximately 500 amino acids long [11, 12]. The β-propeller folds are widely used for protein-protein interaction as well as for catalytic activity itself (reviewed in [13]).

The N-terminal Sema domain in Plxn is followed by three sequential PSI (present in Plxns, Semaphorins and Integrins) repeats and three or four sequential IPT domain

repeats (Immunoglobulin-like fold shared by Plxns and Transcription factors) followed by the trans-membrane-domain [4, 11, 17]. PSI is also called a small cysteine-rich domain (CRD) or MRS (Met-related sequence), and it is shared by MET and RON as well as by most of the Semaphorin family except viral Sema VIII [2, 18]. A putative function for the PSI domain of Plxns was suggested by homophilic binding of Plxns cloned from Xenopus tadpole [2]. Overexpression of Plxn protein in mouse fibroblast L cells caused cellular adhesions to purified Plxn proteins immobilized on plates. Such intercellular aggregation occurred only in the presence of calcium ions, which were partially blocked by Plxn-specific antiserum. These authors ascribed the homophilic interaction of Plxn proteins to serial cysteine-rich PSI domains implying disulfide bridges, although no experimental evidence was provided [2]. This assumption was mainly based upon the evidence that cysteine-rich repeats in the PSI domain of Sema3A (also referred to collapsin-1 or SemD) are responsible for forming a homodimer of Sema3A via a disulfide bond, eliciting its collapse-inducing activity [19, 20]. The crystal structure of the integrin β3 subunit PSI domain revealed its importance in creating a platform for binding an α subunit as well as in regulating ligand-binding [21]. The functions of the PSI in Plxns is still only implied based on the domain-specific functional analyses of other well-studied proteins homologous to Plxns, and no functions of the PSI domain in Plxns has been specifically determined to date [18, 22].

IPT is also referred to as simply Ig domain or TIG (transcription factor Ig domain), similar to the Ig-like domain sequence of the NF-κB family members [13, 23, 24]. Sequential copies of IPT in Plxns are also found in MET and RON, while some of the Sema members (II-V, VII) contain only a single copy of the Ig-like domain [13, 23, 24].

Sequential IPT domains appear to be involved in modulating protein-protein interactions or in protein-binding itself. A monoclonal antibody specific for the PSI domain of β2 integrin did not suppress ligand-binding of the receptor but antibody specific for the putative Ig-like domain adjacent to PSI did block ligand-binding. This suggests that the putative Ig-like domain of β2 integrin is necessary for dimerization of αβ subunits [25]. In other reports, natural splicing variants of RON lacking a region within the IPT domain caused hyperactive kinase activity of the protein, which became oncogenic in human colorectal carcinoma cells, suggesting a regulatory role in controlling the catalytic activity exhibited by other protein domains [26, 27]. More dramatic evidence of IPT domain function was revealed by a mutant Met protein containing serial IPT domains but lacking the Sema domain. The third and fourth IPT domains of Met were sufficient to bind its ligand, hepatocyte growth factor (HGF) with higher affinity, but antagonized HGF-induced invasive growth. The authors proposed a role in which the IPT domains cooperate with the Sema domain in binding to HGF and in controlling HGF-induced Met kinase activity [28]. The potential importance of serial IPT domains in Plxns was shown by its role in binding triggering receptors on myeloid cells 2 (Trem-2), a TREM family member of Ig superfamily, in a Cos-7 cell overexpression system. PlxnA1 interaction with Trem-2 was maintained with the IPT domain of PlxnA1 lacking the Sema and the PSI domains, suggesting that the IPT domain of PlxnA1 contributes to Trem-2-binding [29]. Absolute necessity of the IPT domain for the functional interaction of PlxnA1 with Trem-2 was not proven under physiologically relevant conditions in this report. However, a recently published study provided another example of additional insights into a function of the IPT domain, using another Trem family protein called PDC-Trem on the surface of

plasmacytoid dendritic cells. The authors showed that association between PlxnA1 and PDC-Trem was abrogated in the absence of the IPT domain, while their association was still intact without the Sema domain, suggesting that the IPT domain in PlxnA1 is responsible for cis-interactions with Trem proteins [30].

The cytoplasmic element of Plxns was shown to be essential for transducing signal upon the ligand binding. A spinal neuron over-expressing a truncated mutant of PlxnA1 that lacked the cytoplasmic tail did not respond to Sema3A stimulation even when associated with its co-receptor, Nrp-1 [3, 15, 16]. Unlike MET and RON receptors that contain tyrosine kinase activity, the cytoplasmic portions of Plxns or Semaphorins have not exhibited any kinase activity [31]. Instead, Plxns contain two segmented R-Ras GTPase-activating protein (GAP) domains homologous to p120 RasGAP and SynGAP in their cytoplasmic regions [32]. A region required for binding small GTPases such as Rnd1 or RhoD is located between the GAP domains [32, 33]. The relationship between Plxns and small GTPases will be discussed later.

1.1.2. Semaphorins.

Plxns have been known as receptors for Semaphorin molecules upon which Plxns transduce signals for axonal growth or collapse [3, 4, 11-15, 34, 35]. Semaphorins, primarily referred to collapsins, were first identified in the early 1990s as axonal guidance factors [36, 37]. Since then, more than twenty members of Semaphorins have been identified and categorized into eight subclasses based on their origins and sequence homologies, including invertebrate (Class I and II) Semaphorins, viral (Class VIII) and vertebrate (class III through VII) Semaphorins (Figure 1.2) [38, 39].

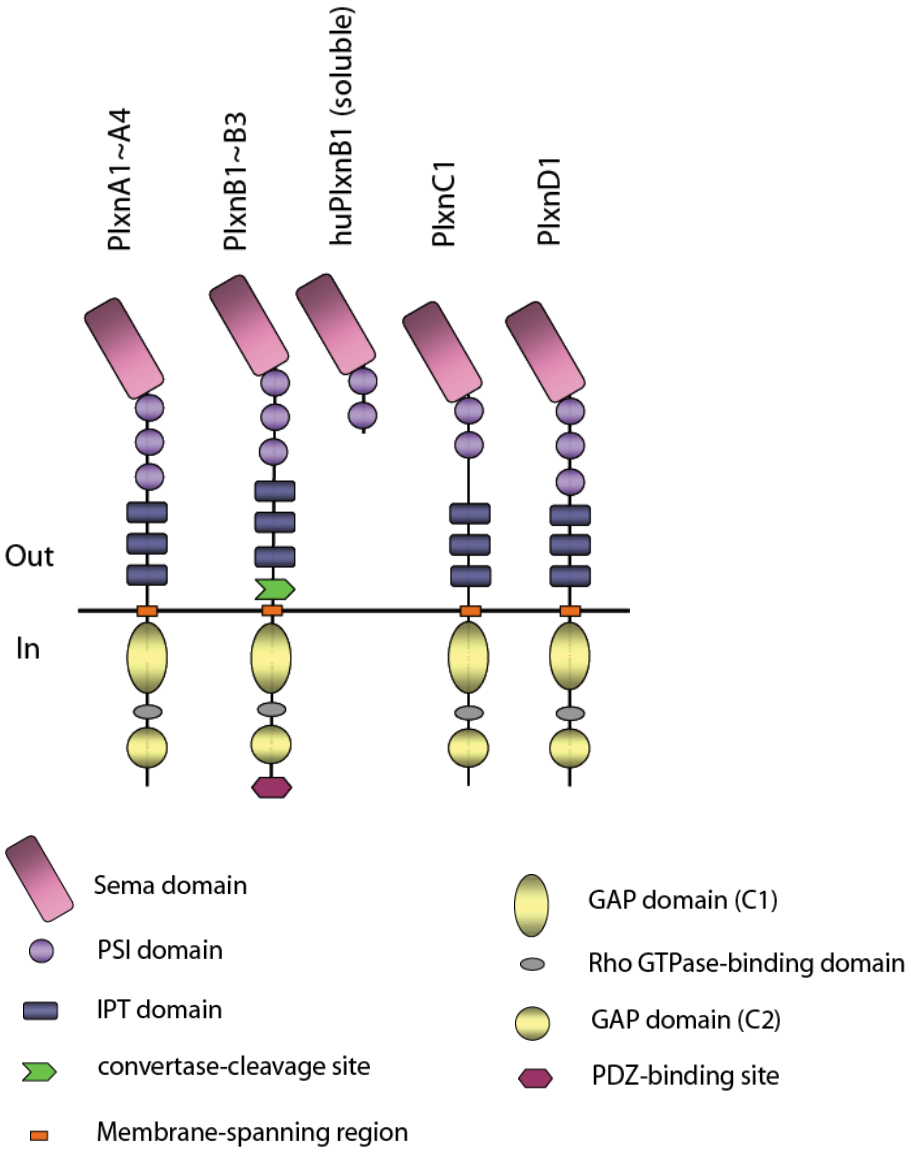


Figure 1.1. Mouse and human Plxn molecules. The Plxn family is divided into four subfamilies designated A through D, and comprises a total of nine members. Soluble huPlxnB1 is a short splice variant that is also found in humans along with full-length PlxnB1.

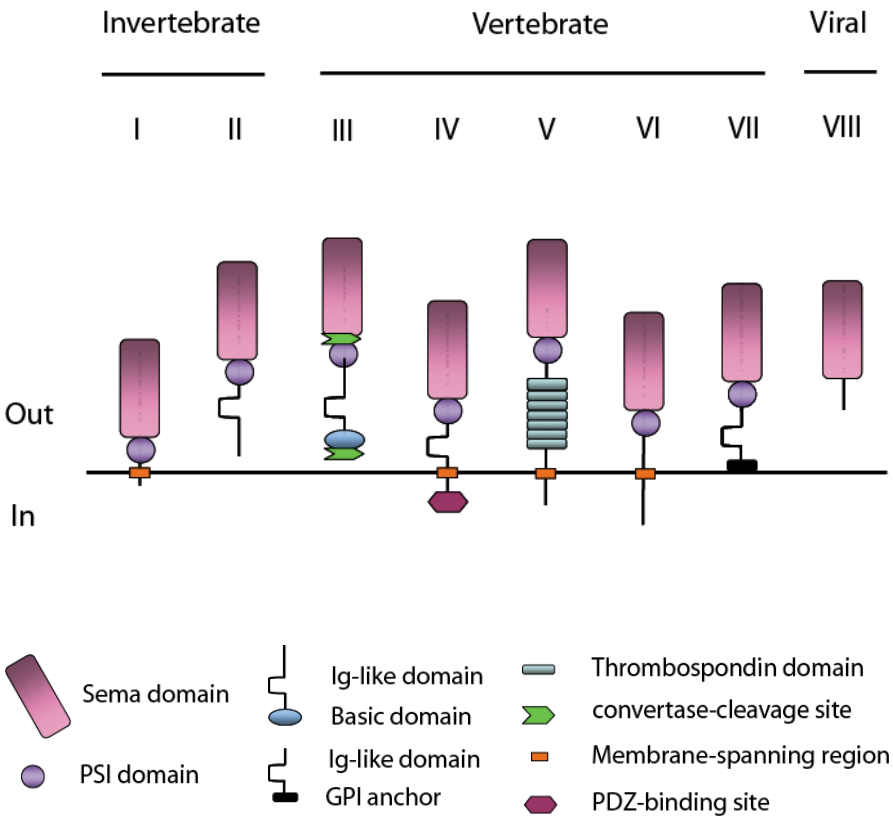


Figure 1.2. Invertebrate (I and II), vertebrate (III~VII), and viral (VIII) Semaphorins. Class II, III, and VIII semaphorins are secreted, but the other members (I, IV-VII) are membrane-bound, either membrane-spanning (IV~VI) or GPI-anchored (VII).

The membrane-bound semaphorins (I, and IV through VII) as well as secreted members (II-III, and VIII) exist, indicating that semaphorin-Plxn interactions might be initiated by trans-interaction between two membrane-bound proteins on adjacent cells; by interaction between soluble secreted ligand and a membrane-bound receptor; or by binding of a soluble secreted ligand and a secreted receptor [3, 9]. One in particular, Sema4D which is categorized as a membrane-bound semaphorin also has a secreted soluble form, generated by alternative splicing or by proteolytic cleavage upon cellular stimulation [40-44].

Particularly, Class III Semaphorins require two receptor components, one of which is a Plxn and the other, a Neuropilin. Neuropilins funtion to facilitate high-affinity binding to the ligand. The co-receptor, Plxns are responsible for transducing signals through the cytoplasmic tail [3, 4, 15, 19, 32, 34, 45-49]. An exception is Sema3E, for which PlxnD1 is sufficient for binding and a signaling response [50]. However, more recent evidence suggests that some Semaphorins utilize not only Plxns and Nrps, but proteins such as integrins, CD72 or TIM-2, as their receptors [51-54]. The molecular interactions between Plxns and semaphorins are therefore much more complex than have been observed thus far.

Semaphorins have been shown to function as either attractive or repulsive guidance cues, even in the same neuron [55-57]. For example, an identical cortical explant from rat embryos showed opposing responses, upon encountering two different Semaphorins: The explants exhibited growth cone collapse upon Sema3A (known previously as SemD), but axonal outgrowth in response to Sema3C (SemE) [55]. Furthermore, Sema3A is also capable of inducing axonal outgrowth, although it is well established to cause growth cone collapse. One of the factors that affect the cellular response to Sema3A appears to be the cellular level of cyclic nucleotides, leading to either growth cone collapse or axonal outgrowth [56]. The availability of their coreceptors could also be a switch between attractive and repulsive signaling, in the presence of the same ligand. Neurons expressing both PlxnD1 and Nrp-1 were attracted to Sema3E, but neurons expressing PlxnD1 alone were repelled from it [58]. Sema6D has also been shown to induce completely opposite phenotypes through PlxnA1 depending on which co-receptor of PlxnA1 is evidenced by VEGFR2 and Off-track in cardiac morphogenesis of chick

embryonic development [59]. Thus, it appears that a single Semaphorin can generate differential signals depending on the cellular contexts. Beyond the original observations, it is now well understood that stimulation of Plxns with Semaphorins is not only implicated in neuronal networking but also in regulating immune responses as well as cell migration and tumorigenesis in various tissues [29, 30, 40, 52, 54, 60-77].

In the immune system, multiple Plxns and semaphorins have been functionally recognized, although this area of research is still in its infancy. PlxnA1 is expressed in mature dendritic cells (DCs) [29, 71, 73, 78]. And PlxnA1 has been shown to bind Sema6D which is expressed on T lymphocytes, stimulating T cell activation as demonstrated by our group and others [29, 71, 78, 79]. PlxnA4 is also detected in DCs, where its functionality is still unclear [73]. However, in T cells, it is recognized as a negative regulator of T cell responses by employing *plxna4*$^{-/-}$ mice. From the report, PlxnA4 is suspected to cooperate with Nrp-1 in order to respond to Sema3A stimulation in T cells, in that the hyper-proliferative phenotype of PlxnA4$^{-/-}$ T cells is similar to that of Nrp$^{-/-}$ T cells or Sema3A$^{-/-}$ T cells [73]. On the other hand, PlxnB1 and C1 appear in human immature DCs and monocytes, respectively, and both respond upon engagement with Sema4D (CD100), previously reported as a ligand for CD72 on B cells [80, 81]. It is interesting that dramatic downregulation of PlxnC1 funtions parallel to induction of PlxnB1 upon *in vitro* DC maturation. In the report, PlxnC1-expressing monocytes and PlxnB1-expressing immature DCs showed reduced migration when Sema4D was added, whether their migration was spontaneous or chemokine-induced. Such inhibition of migration by Sema4D ligation suggests that Sema4D suppresses migration of monocytes or immature DCs through either PlxnC1 or PlxnB1, respectively [81]. In contrast,

PlxnC1 is also reported to be found on mature DCs, where it is inhibitory for integrin-mediated DC adhesion and migration upon poxviral Sema A39R, using *plxnc1*$^{-/-}$ mice [69, 70]. A discrepancy in expression patterns of PlxnC1 in these reports may be due to the differences between humans and mice. Meanwhile, interaction of PlxnB1 with Sema4D has also been identified to be crucial for B cell survival and proliferation [60, 82]. Given these reports, it is clear that each Plxn has multiple ligands, some of which are shared by other Plxns. As a result, the functional outcome of each Plxn would then be dependent on the spatiotemporal availability of a particular ligand. The consequences of these molecular interactions and signal transductions are therefore expected to be much more complex than initially presumed. The importance of Plxns and Semaphorins in other systems with cumulative findings of their functions is reviewed by others [82-84].

1.2. PlxnA1 and small GTPases

Small GTPases (G-proteins) have been implicated in Nrp-1/PlxnA1-mediated Sema3A response in neurons, and their signaling to the actin cytoskeleton for axonal guidance. Indeed, multiple small GTPases that directly or indirectly associate with PlxnA1 have been identified [3, 32, 33, 85-90]. Our Plxn study was performed in the context of DC-T cell conjugation. Although the mechanism regulating DC cytoskeleton is not fully understood, cytoskeletal organization and integrin activation upon DC-T cell conjugation are known to be critical for activation of both participants [91-93]. Thus, it is important to include a discussion of PlxnA1-associated small G-proteins.

1.2.1. ***Rho GTPases:***

PlxnA1 has been linked to Rho or Rho-like GTPases. PlxnA1 overexpressed in HEK 293T cells has been found to physically interact with both Rnd1 and RhoD as shown by GST pull-down assays [33]. Rnd1 is known as a constitutively active member of the Rho GTPase family [87, 94]. Rnd1 and RhoD appear to either pass Sema3A-induced collapse signal or block it, respectively. PlxnA1 signaling upon Sema3A engagement is suggested to be balanced by these two players. None of the other Rho-related GTPases tested such as Rac1, Rnd2 or RhoG seems to form a complex with overexpressed PlxnA1 protein, in GST pull-down assays [33]. In general, studies have shown that Rnd1 activation signals the disassembly of actin cytoskeleton and Rnd1 regulates actin cytoskeleton by antagonizing RhoA activity, for example, via activation of RhoGAP proteins [87, 95-98]. Rho and Rac are known to activate actin polymerization through the following mechanism. Rho and Rac both activate LIM-kinase through their downsream kinase, ROCK and PAK, respectively [86, 99, 100]. In turn, LIM-kinase phosphorylates and inactivates cofilin, an actin-depolymerizing factor, thereby activating actin polymerization [101-103]. Taken together, actin filaments are depolymerized for the retraction of lamellipodia and filopodia in collapsed growth cones, explaining a role of Rnd1 in the collapse response [33, 85, 104]. Interestingly, recruitment of Rnd1 to PlxnA1 was important for its role in facilitating the collapse response upon Sema3A [33]. This is partly due to the fact that RhoD binds to the overlapping site in PlxnA1 protein. Thus, RhoD is thought to antagonize Rnd1 by competing for the binding site on PlxnA1 or by changing a conformation of the Rnd1-binding region of PlxnA1 [33]. Rnd1 has also been implicated in R-Ras signaling for integrin activation as well as for the collapse response in the context of interaction with Plxns [105-107]. Rnd1 requirement for the Sema3A-induced

collapse response will be further discussed related to the regulation of R-Ras activity by PlxnA1.

Regarding RhoA and Rac1, the same group showed earlier that neither RhoA nor Rac1 specifically interacts with PlxnA1 [32]. However, other investigators reported subsequently that overexpressed Rac1 physically interacts with co-expressed PlxnA1 and is required for PlxnA1 signaling for cell collapse upon Sema3A engagement in COS cells [90]. This has been reproduced in dorsal root ganglia and spinal motor neurons by others [89, 90, 108, 109]. However, the collapse induced by a constitutively active form of PlxnA1 does not seem to require Rac1 activity, suggesting that Rac1 is not located downstream of PlxnA1 in the signaling cascade [90]. Regarding discrepancies among reports, it should be noted that most of the studies have been performed in certain model systems using proteins overexpressed in cell lines.

Collapsin response mediator protein (CRMP) is another family of molecules that is physically associated with PlxnA1 and implicated in Sema3A-induced axonal repulsion [110, 111]. Overexpressed CRMP2 is shown to be tightly associated with co-expressed PlxnA1 upon Sema3A stimulation, leading to facilitated Sema3A-induced cell contraction [110]. CRMP2 is primarily related to regulation of microtubule dynamics, as well as functioning as a substrate of Rho kinase, to binding actin filaments [112-114].

In summary, Rho and Rho-like small GTPases including RhoA, Rac1, Rnd1, and RhoD, seem to interact with PlxnA1 directly or indirectly. In general, they function in regulating cellular behaviors such as cell adhesion and motility through rearrangement of the actin cytoskeleton upon Sema3A stimulation by interacting with PlxnA1. In fact, Rho and Rho-like G-proteins are analogous in their underlying mechanism of action resulting in similar

phenotypes, even though they have been recognized for their distinct roles depending on cell types and cellular settings. Furthermore, PlxnA1 has also been shown to induce completely opposite phenotypes upon the same stimulus, depending on its available co-receptor or on the cellular level of cyclic nucleotides, suggesting that there are cellular mechanisms regulating multiple mechanisms [56, 59]. Thus, it is likely that PlxnA1 utilizes parallel and distinct pathways by employing a differential Rho GTPase in a particular context.

1.2.2. *R-Ras and Rap GTPases:*

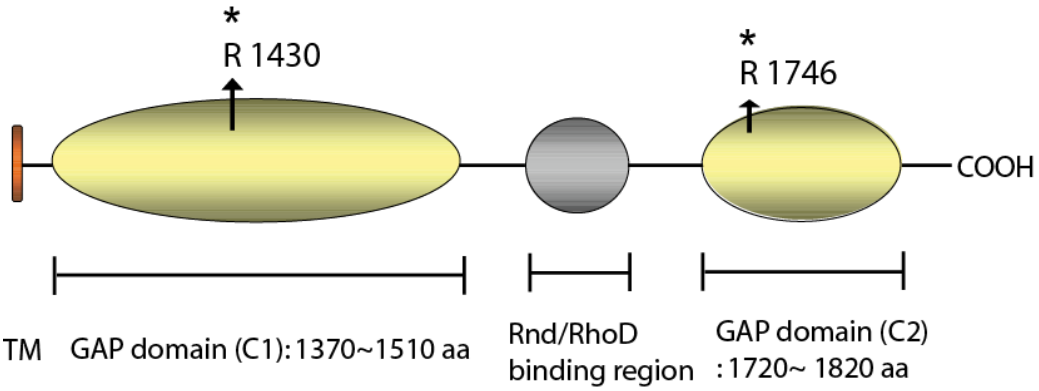


Figure 1.3. The cytoplasmic region of PlxnA1 from the membrane-spanning sequence (TM) through domains homologous to SynGAP (C1) and R-RasGAP (C2). Critical Arg (R) residues at position 1430 and position 1746 in amino acid sequence of PlxnA1. Rnd1 and RhoD bind to PlxnA1 between C1 and C2.

The activity of Small GTPases including Rho GTPases as well as the Ras superfamily is regulated by their own guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs activate G-protein signaling by facilitating the exchange reaction from G-protein-bound GDP to GTP. In contrast, GAPs dampen the signaling of G-protein by facilitating G-protein-bound GTP hydrolysis. These regulatory elements of individual G-proteins are critical for controlling the activity of specific GTPases by which cellular

behaviors such as cell adhesion or movement are regulated under certain physiological conditions (reviewed in [115]).

PlxnA1 has been linked to regulation of R-Ras function through its potential activity of GTPase activating protein (GAP) based on its sequence homology [32, 105]. A Ras-related small GTPases, R-Ras is highly homologous to human Ras (H-Ras) [116]. Despite the sequence similarity, however, R-Ras has been demonstrated to play a distinct role from that of protooncogenic H-Ras [117, 118]. R-Ras activates integrin signaling for cellular migration and adhesion [119]. Notably, PlxnA1 contains segmented R-Ras GAP conserved regions including critical R residues essential for the GAP activity, in its cytoplasmic region (Figure 1.3) [32]. Instigated by the sequence homology of PlxnA1 to R-RasGAP, several studies have sought to reveal GAP activity of PlxnA1 for R-Ras. Mutations of conserved R residues (R1430 and R1746) in the GAP region of PlxnA1 completely abolished Sema3A-induced collapse in COS-7 cells, suggesting R-RasGAP activity of PlxnA1 is required for a Sema3A-induced response.

However, direct evidence of GAP activity by PlxnA1 has not been forthcoming [32]. It is PlxnB1 that has displayed direct evidence of GAP activity for R-Ras [105]. This study revealed that R-RasGAP conserved regions in PlxnB1 are required for its GAP activity specifically for R-Ras. Rnd1-binding to PlxnB1 is also required for its R-Ras GAP activity, such that PlxnB1 performs R-Ras GAP activity in response to Sema4D engagement, only when Rnd1 is provided. According to the report, endogenous PlxnB1 naturally signals for neurite retraction upon Sema4D but overexpressed mutant PlxnB1 lacking critical R residues does not facilitate R-Ras GTP hydrolysis but suppresses Sema4D-induced neurite retraction in PC12 cells [105]. A subsequent report from the same group shows that PlxnB1 suppresses

R-Ras-mediated activation of β1 integrin upon Sema4D, leading to a reduction of integrin-mediated cell migration [107]. In general, it has been known that R-Ras plays a crucial role in cellular adhesion by stimulating integrins, which in turn promote cell adhesion and migration, as mentioned earlier [119-121]. Interesting enough, R-RasGAP activity of PlxnB1 suppresses such cellular behaviors [105, 107].

Contrary to PlxnB1 exhibiting R-Ras GAP activity, direct evidence is still lacking that PlxnA1 performs this function [105]. It has been implied that Plxn-A1 signaling in response to Sema3A is linked to growth cone collapse through its possible R-RasGAP activity, analogous to PlxnB1 effect upon Sema4D [105]. In line with previous findings that mutations in the GAP domain of PlxnA1 suppress the Sema3A-induced repulsion, this report demonstrates that downregulation of R-Ras activity is also required for the Sema3A-induced growth cone collapse. Introduction of a constitutively active R-Ras mutant into rat hippocampal neurons suppresses growth cone collapse upon Sema3A stimulation [32, 33, 105]. Another report suggests a link between PlxnA1 and Rac as well as Rnd1 and R-Ras [122]. The report demonstrates that a molecule called FARP2 (a RacGEF), which is associated with PlxnA1 in a resting state dorsal root ganglion, becomes dissociated from PlxnA1 to activate Rac, upon Sema3A stimulation. In turn, Rnd1 is recruited to PlxnA1 upon Sema3A to stimulate the R-RasGAP activity of PlxnA1, resulting in axonal repulsion [122]. Although direct proof of PlxnA1 GAP activity for R-Ras is still lacking and it has been indirectly implied only in the context of Sema3A stimulation along with Nrp-1-PlxnA1 association, GAP activity of PlxnA1 for R-Ras has been presumed based on such a change in cellular behaviors [105, 122].

A Ras-like small GTPase, Rap1 was introduced as another possible player upon PlxnA1 signaling. Rap1 is activated by R-Ras for integrin stimulation in a macrophage-like cell line [123]. Analogous to R-Ras, Rap1 has been implicated in translation of environmental stimuli into integrin-mediated cellular adhesion and motility in immune cells, among others [124-132]. In particular, Rap1 has been shown to play a key role in chemokine-induced DC trafficking. The importance of Rap1 in DC migration is revealed from a mouse model deficient in a Rap1 effector molecule RAPL (Regulator of cell Adhesion and Polarization enriched in Lymphoid tissues), where DCs are impaired in migration towards the draining lymph nodes [125]. Furthermore, Rap1 activity has been found to be required for BCR and LFA-1-mediated activation of B cells which are professional antigen-presenting cells (APC) [133]. Also, in activating T cells, Rap1 plays a key role in regulating both T cell-APC interactions and T cell receptor-mediated T cell responses through LFA-1-ICAM-1 interaction.

However, fine tuning of Rap1 activity seems critical for overall T cell response [129, 131]. Rap1 is transiently activated upon T cell receptor stimulation through calcium influx, and consequently elevated Rap1 activity strengthens the affinity between LFA-1 and ICAM-1 upon T cell engagement with antigen-presenting cells (APC) [129]. CD3 stimulation increases Rap1 activity, while CD28 dampens it, thereby modulating LFA-1 affinity to ICAM-1, although both CD3 and CD28 are known to induce calcium influx upon T cell activation [131, 134, 135]. However, a constitutively active Rap1 antagonizes Erk activation induced by antigen-dependent TCR stimulation but also causes extensively elevated levels of $p27^{kip1}$, an indicator of cell cycle arrest, with subsequent reduction of IL-2 levels. Overexpression of a dominant negative Rap1 or a RapGAP also reduces the stability of T

cell-APC conjugates, and subsequent IL-2 production [129]. CD3 and CD28-mediated precise modulation of transient Rap1 activity is critical for optimal T cell activation, by maintaining stable T cell-APC conjugation via optimal LFA-1-ICAM-1 interaction [129, 131]. Since strict regulation of appropriate Rap1 activity appears to be critical for optimal T cell response, some of the T cell stimulatory factors might also down regulate Rap1 activity for precise control of its optimal levels. Collectively, Rap1 is shown to be involved in T cell activation as well as in DC trafficking.

Our original observations indicate that PlxnA1 on DCs is essential for cognate T cell activation, in which R-RasGAP activity of PlxnA1 is presumably involved. In addition, Rap1 seems to be activated by R-Ras, facilitating our hypothesis that Rap1 activity is regulated by PlxnA1 upon T cell conjugation [71, 105, 123]. In accordance with our observations described in Chapter II, we are currently seeking to determine whether Rap1 activity is regulated by PlxnA1. Rap1 and R-Ras may be sequentially operational upon T cell engagement, as suggested by a report that Rap1 is downstream of R-Ras signaling towards $\alpha_M\beta_2$ integrin activation in a macrophage-like cell line [123]. Another possibility is that PlxnA1 might display a GAP activity for Rap1 without involving R-Ras, since PlxnA1 contains a SynGAP conserved region essential for GAP activity [32, 136]. Such a possibility is also supported by observations that Rap1 and R-Ras share some of their GAP proteins [137].

1.3. PlxnA1 in the immune system:

Plxns function in a spatiotemporally orchestrated manner, as is evident from the differential expression of the A subfamily of Plxns in the mouse nervous system [138,

139]. Coordinated Plxn activity becomes even more complex when considering the multitude of combinations and/or differential availability of their multiple co-receptors and/or ligands [15, 29, 34, 51, 59, 140]. It has been well-documented that PlxnA1 responds to Sema3A stimulation, in the nervous system. On the surface of neurons, PlxnA1 forms a receptor complex with Nrp-1 resulting in high affinity binding of Sema3A. This results in the transduction of PlxnA1 signals through its cytoplasmic tail, causing growth cone collapse in neuronal development [9, 10, 34, 141]. As mentioned previously, PlxnA1 signaling upon Sema3A can transduce either attractive or repulsive depending on the context [56, 142]. Nevertheless, the critical role of PlxnA1 in the nervous system is evident from impaired neuronal networking in the absence of PlxnA1.

In previous studies by our group involving the regulation of genes by CIITA in mouse bone marrow-derived DCs, we observed a strong correlation between CIITA and the regulation of PlxnA1, using Affymetrix analysis [71]. CIITA is the master regulator governing the expression of MHC class II molecules that present antigens to cognate T cell receptor (TCR) on $CD4^+$ T helper cells, in order to initiate antigen-specific T cell-mediated adaptive immunity [143-145]. In the absence of CIITA, transcripts of PlxnA1 along with MHC class II are very low in DCs [71]. Regulation of PlxnA1 by CIITA during DC maturation has several important implications: 1) CIITA governs adaptive immunity by regulating expression of MHC class II molecules, without which antigen-specific T cell immunity would be impaired, as exemplified by the human genetic disorder, Bare Lymphocyte Syndrome [143, 146, 147]; 2) DCs that highly express PlxnA1 are the sentinels for host immune response against pathogens and are required for vaccine-induced immunity including the vaccines targeting cancers [148, 149]. DCs are

the main mediators of the innate-adaptive immunity transition, and also are the only known cell type that is capable of activating naïve T lymphocytes, initiating T cell-mediated adaptive immunity [150-154]. Indeed, based on our own studies, PlxnA1 on DCs is critical for DC function in activating naïve T cells. A DC-like cell line shows reduced capacity to activate cognate T cells upon *in vitro* depletion of PlxnA1, as revealed by significantly less secretion of IL-2, a pro-inflammatory proliferative cytokine [71]. Therefore, it will be of interest to determine whether PlxnA1 is required for the initiation of T cell response *in vivo*, including the step in which PlxnA1 is required for T cell activation by DCs and the mechanisms by which PlxnA1 exerts its role.

Candidates for PlxnA1 ligand in the immune system include Sema3A primarily identified in the nervous system, and Sema6D studied in cardiovascular development of chick embryos [15, 29, 34]. In the cardiovascular system, PlxnA1 forms a complex with vascular endothelial growth factor receptor 2 (VEGFR2) or with Off-track, leading to a differential phenotype, upon Sema6D stimulation [59]. Sema6D stimulation results in augmented migration of outgrowing cells from the conotruncal segment of cardiac explants of chick embryos where PlxnA1 is associated with VEGFR2, while Sema6D treatment lessens migration of those from the ventricle segment where PlxnA1 is interacting with Off-track. Taken together, it is evident that PlxnA1 can respond to a multitude of ligands, for each of which it may function as a receptor or pair with additional co-receptors, in a spatiotemporal manner [15, 59]. Thus, to understand the role of PlxnA1on DCs, it is imperative to determine the ligand for this receptor on T lymphocytes.

1.4. BMDCs and antigen-presentation to $CD4^+$ T lymphocytes.

DCs are a highly heterogeneous cell type in terms of developmental lineages, phenotypes, and functionalities. For example, there are currently three known subsets of DCs that reside in the mouse spleen [155-158]: myeloid DCs (mDCs: $CD11c^+$ $CD11b^+$ $CD8\alpha^-$); lymphoid DCs (lDCs: $CD11c^+$ $CD11b^{low}$ $CD8\alpha^+$); and plasmacytoid DCs (pDCs: mPDCA-1^+ $B220^+$) [155-158]. Other immune tissues seem to have comparable subsets of DCs [155, 157, 158]. Each subtype of DCs shares CD11c as a common surface marker, but myeloid DCs (mDCs) lack CD8α and CD205 surface expression whereas lymphoid DCs (lDCs) express CD205 along with the CD8α homodimer analogous to thymic DCs [159]. $CD8\alpha^-$ mDCs can be generated *in vitro* from bone marrow myeloid precursor cells, but $CD8\alpha^+$ lDCs and T cells develop from their common thymic lymphoid precursors [160-163]. It was reported that $CD8\alpha^+$ DCs had not been reproducibly generated from $CD8\alpha^-$DCs, suggesting that these two subtypes might be derived from unique lineages [163, 164]. Thus, $CD11c^+$ DCs were initially considered to have two distinct lineages, mDCs and lDCs. However, their ontogenies became vague when common myeloid progenitors were found to develop into $CD8\alpha^+$ DCs [165]. Furthermore, both $CD8\alpha^-$ and $CD8\alpha^+$ DCs could also be generated from lymphoid precursors from the thymus or the spleen [166]. Thus, the definitions of 'myeloid' lineage of $CD8\alpha^-$ DCs versus the 'lymphoid' lineage of $CD8\alpha^+$ DCs are now under dispute. Even though the patterns of their surface marker expression cannot represent their specific lineage, at least their localization patterns are distinctive. It has been shown that $CD8\alpha^+$ lDCs reside in the T cell area of the spleen and thymic medulla, inducing tolerance by suppressing T cell response against self-antigens most likely derived from

dying cells. In contrast, $CD8\alpha^-$ mDCs are located either in the marginal zone of the spleen or in the periphery and take up foreign antigens and migrate to the T cell area to initiate T cell responses [167]. These distinct functions correlating with their localization appear to be supported by independent findings that $CD8\alpha^-$ mDCs have superior phagocytic capacities compared to $CD8\alpha^+$ lDCs [157, 168, 169]. Other studies suggest, however, that both subsets of DCs are stimulatory but that the T cell responses elicited by either subset appear unique. According to the authors, $CD8\alpha^+$ DCs present antigens on both MHC class I and II molecules, leading to CTL expansion and $CD4^+$ T cell response, respectively [170-172]. In contrast, $CD8\alpha^-$ DCs primarily expand $CD4^+$ T cell response [173]. However, both subsets of DCs have also been demonstrated to equally induce antiviral protective CTL response, *in vivo* [174]. Debates are still continuing on T cell polarization to either Th1 or Th2 by either subset. Several studies have shown that $CD8\alpha^+$ lDCs and $CD8\alpha^-$ mDCs polarize $CD4^+$ T cells to Th1 and Th2 responses, respectively, attributed to the higher amount of IL-12p70 produced by $CD8\alpha^+$ lDCs [173, 175-179]. Th1-inducing interferon-γ (IFN-γ) production by T cells mainly depends on the amount of IL-12p70 [176, 180, 181]. However, others have witnessed that $CD4^+$ T cells produce comparable levels of Th1 and Th2 cytokines, upon *in vivo* activation by either subset of DCs [182]. Investigations are still continuing with a recent publication showing that $CD8\alpha^-$ mDCs and $CD8\alpha^+$ lDCs are both capable of inducing Th1 response by producing interferon-γ (IFN-γ) but through differential mechanisms. IFN- γ production by $CD8\alpha^-$ mDCs is IL-12p70-dependent but that of $CD8\alpha^+$ lDCs does not appear to be [183]. Thus, these two subsets seem adaptable in inducing an appropriate T cell response but either subset may respond more efficiently depending on the antigenic

route [182]. BMDCs generated with GM-CSF and IL-4 along with TNF-α have been considered similar to mDCs in terms of morphological features including very low levels of CD8α [71, 161, 184].

On the other hand, a recently identified subset, mPDCA-1^+ pDCs represent a third category in DC subsets [185-187]. Murine pDCs express distinct surface markers such as B220, Ly-6C, and mPDCA-1, although pDCs express CD11c, and also CD8α upon activation [185, 186, 188-190]. pDCs are enriched by Flt3-Ligand *in vivo* as well as *in vitro* from mouse bone marrow precursors [191, 192]. Functionally, pDCs are poor at stimulating naïve T cells [193]. Because of this weak T cell-priming capacity, pDCs used to be considered immature DCs or DC precursor cells [185, 192-194]. pDCs are rather tolerogenic, since they perform strong inhibitory activity against antigen-specific T cell response, primarily by inducing regulatory T cells from naïve T cells, in some cases [195, 196]. Instead, pDCs are devoted to producing high levels of Type I IFN upon viral challenge, inducing antiviral immunity. As such, pDCs are also referred to IFN-producing cells [197]. Differences between murine and human pDCs and their TLR-mediated innate immune properties are a different subject and reviewed elsewhere [188, 198].

Mature DCs are the most potent professional antigen-presenting cells (APCs) comprising a unique cellular repertoire that performs the critical role of activating naïve T cells, resulting in the initiation of T cell-mediated adaptive immunity [199]. Under unstimulated conditions, however, DCs remain in an immature state, where they have high phagocytic capacity but very low ability to initiate T cell response. This is a consequence of the cells neither being equipped with MHC molecules to load antigens,

nor with costimulatory molecules necessary for T cell stimulation [200, 201]. Thus, in case immature DCs encounter naïve T cells, they induce T cell anergy leading to immune tolerance instead of T cell-mediated immunity [202, 203]. *In vivo*, immature DCs circulate in the blood and reside in peripheral tissues where they become mature and activated upon encountering pathogens and/or inflammatory cytokines (e.g.TNF-α) [175, 204-207]. Maturation of DCs leads to high levels of MHC class II molecules as well as costimulators resulting in T cell activation and a subsequent immune response rather than tolerance [199, 200].

In order to activate naïve T cells located in the secondary lymphoid organs, DCs in the periphery must take up antigens, process them, and present the resulting peptide antigens on surface MHC molecules. These DCs that have taken up antigens become mobilized and migrate from the inflamed tissue to the draining lymph nodes (DLNs) where they encounter naïve T cells. DCs are mobilized in the periphery by inflammatory cytokines including TNF-α, resulting in CCR7 upregulation on the cells. CCR7 functions as a receptor for chemokines CCL19 and CCL21 that guide DCs to the DLNs [208-210]. CCR7 is known to be critical for DC migration, since $CCR7^{-/-}$ Langerhans cells are unable to migrate from the skin to the DLNs even in the presence of normal levels of chemokines [211]. Ultimately, upon DC-T cell engagement, DCs should contain the operational ability including MHC molecules presenting peptides to activate T cells. Such potential to activate T lymphocytes is gained during the migration and maturation of DCs on their way to T cell areas [208, 210, 212]. DC function, therefore, encompasses a multitude of events that culminate in their ability to activate T cells and initiate an adaptive immune response.

1.5. Our research on PlxnA1 on the surface of BMDCs

Our initial study suggested that PlxnA1 on mature DCs is essential for T cell stimulation [71]. The mechanism by which PlxnA1 transduces a signal upon T cell conjugation was to be defined. As summarized, small G-proteins and cytoskeletal elements were expected to be involved in the PlxnA1 pathway. Our laboratory has investigated PlxnA1 in the context of DC surface function upon cognate T cell conjugation, to determine whether and what small G-proteins would be regulated by PlxnA1. One of the PlxnA1 ligands present on T cells has been identified. More importantly, based on independent studies, it is evident that PlxnA1 interaction with its ligand not only contributes to T cell-priming but also stimulates DCs for further activation of T cell response [29, 79]. Since PlxnA1 has been implicated in the model inflammatory disease, experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) peptide, it would be valuable to understand what T cell responses DC cell surface PlxnA1 induces. This information will foster the development of therapeutics in modulating PlxnA1 function in excessive inflammatory conditions [29].

The work presented here indicates that PlxnA1 plays a dual role in T cell priming. PlxnA1 is not only shown to be crucial in stimulating naïve T cells upon conjugation, but also in chemokine-induced DC migration. Moreover, we have found that PlxnA1 is important for rearrangement of actin filaments through small GTPases in DCs towards the interface with T cells upon contact, which is crucial for T cell activation. In the

absence of such a stimulatory role for PlxnA1 in the initial stages of T cell activation, subsequent T cell proliferation is demonstrated to be significantly reduced.

CHAPTER II: RHO ACTIVATION AND ACTIN POLARIZATION ARE DEPENDENT ON PLEXIN-A1 IN DENDRITIC CELLS

Abstract

We recently identified expression of the semaphorin receptor, PlxnA1, in dendritic cells (DCs); however, its function in these cells remains to be elucidated. To investigate function and maximize physiological relevance, we devised a retroviral approach to ablate PlxnA1 gene expression using small hairpin RNA (shRNA) in primary bone marrow-derived DCs. We show that PlxnA1 localizes within the cytoplasm of immature DCs, becomes membrane-associated and is enriched at the immune synapse in mature DCs. Reducing PlxnA1 expression with shRNA greatly reduced actin polarization as well as Rho activation without affecting Rac or Cdc42 activation. A Rho inhibitor, C3, also reduced actin polarization. These changes were accompanied by the near-ablation of T cell activation. We propose a mechanism of adaptive immune regulation in which PlxnA1 controls Rho activation and actin cytoskeletal rearrangements in DCs that is associated with enhanced DC-T cell interactions.

Introduction

Dendritic cells (DCs) are the most potent type of antigen-presenting cells (APCs), and the only APCs capable of initiating primary immune responses via presentation of antigen in the context of major histocompatibility complex (MHC) class II molecules [175, 213]. Following initial antigen encounter, formation of a physical site known as the "immune synapse" between a T cell and DC is required for initiation of the adaptive immune response. The immune synapse shares similar characteristics with the neuronal synapse, including expression of semaphorins, plxns and neuropilins [214, 215]. Semaphorin family proteins were first observed in the central nervous system (CNS) where they mediate repulsive and attractive axon guidance cues during neural development [3, 216]. Semaphorins are both secreted and membrane-bound, and they are recognized by Plxn family receptors [3, 15, 217, 218]. Plxns mediate cytoskeletal rearrangements that can result in either axon extension or retraction via interaction of their conserved C-terminal Plxn domain with Rho family GTPases [39, 219]. A generally held hypothesis is that developing neurons may utilize plxns to regulate their cytoskeleton and the formation of synapses. However, most studies have relied upon overexpression system in cell lines, whereas regulatory mechanisms of PlxnA1 in primary cells, neuronal or otherwise, are underexplored.

In the immune system, semaphorins, Plxns and neuropilins regulate various activities, including regulation of T cell activation, B cell signaling, monocyte cytokine production and regulation of DC migration [49, 53, 69, 71, 220-226]. We previously demonstrated that the inhibition of PlxnA1 expression in a transformed DC cell line reduced T cell activation [71, 227]. However, the mechanism through which PlxnA1 regulates BMDC interactions with T

cells has remained largely unexplored. We report the novel investigation of PlxnA1 function, involving Rho family control of the actin cytoskeleton, in regulating DC-T cell interactions.

Materials and Methods

Mice: All experiments were performed with 8-12 week old C57BL/6 mice from Jackson Laboratory. OT-II mice, which express the $OVA_{323-339}$-specific TCR transgene on the C57BL/6 background, were obtained from Dr. Michael Croft, (La Jolla Institute of Allergy and Immunology, La Jolla, CA). All animal procedures were conducted in complete compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and are approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill.

Cells: Murine BMDCs were isolated and cultured as previously described [184]. T cells from OT-II mice were isolated from the spleen and purified by negative selection with T enrichment columns (R&D systems).

Conjugation Assay, C3 treatment and Immunofluorescence confocal microscopy: DCs at Day 12 were harvested and pulsed overnight with 50 μg/ml ovalbumin (OVA) (Sigma-Aldrich). DCs (1×10^6) were washed with PBS and combined with an equal number of spleen T cells from OT-II transgenic mice in 100 μl of medium. Cells were pulled down in microtubes by centrifugation at 1,500 rpm for 10 seconds and incubated at 37°C for 45 min. in a water bath. Following conjugation, 1×10^5 cells in 100 μl of PBS were added onto each poly-L-lysine coated coverslip (BD Biosciences), fixed in 4% paraformaldehye (PFA) for 15 min., permeabilized in 0.3% saponin for 5 min., and blocked in 5% BSA in PBS for 30 min. Cell conjugates were stained with antibody for 1 hr. at room temperature. For staining actin

cytoskeleton, Alexa 647-conjugated phalloidin was used. Coverslips were mounted with FluorSave (Calbiochem). Images were captured with the Fluoview FV500 laser scanning confocal microscope (Olympus). C3 exoenzyme specifically inhibits Rho GTPase activity. C3 (10 μg/ml) was added into DC culture from Day 11.5 to Day 12 and during OVA-pulse, for a total of 24 hr. After washing, DCs were harvested and conjugated with OT-II T cells before confocal microscopy analysis. Phalloidin-Alexa Fluor 647 was used to stain actin filaments.

Analysis of Actin polarization at the Immune Synapse: Double blind studies were performed to analyze prepared slides of actin immune synapse polarization using wild-type, PlxnSh, or CtrlSh treated DCs. Actin polarization was determined in reference to control polarized and non-polarized samples. For each treatment group, 70 cells were analyzed and counted. The percentage of cells displaying actin polarization was enumerated. Actin fluorescence intensity at the interface of conjugating cells was quantified via Image J analysis [228]. Briefly, fluorescence intensities of total and interface area were calculated, respectively, for each sample. The fluorescence intensity at the interface was then compared with the total fluorescence for each cell and represented graphically.

Flow Cytometry: Flow cytometry was performed on wild-type, PlxnSh, and CtrlSh virus tansduced DCs at Days 10 and 12, as [71]. Staining was quantified with a BD FACSCalibur.

Assays for GTP-bound Rho, Rac and Cdc42: Prior to conjugation, OT-II T cells were fixed in 4% PFA for 1 min. and washed three times before incubation with an equal number

of OVA-pulsed DCs for 20 min. at 37°C. Following conjugation, DCs and T cells were washed with PBS and lysed in Nonidet P-40 lysis buffer (2×10^7/ml). Rho, Rac and Cdc42 assays were performed according to the manufacturer's instructions using Rhotekin- or PAK-bound agarose beads provided through a gift from Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC), or purchased from Upstate Biotechnology [229, 230]. Lysate controls were blotted for total Rho, Rac or Cdc42 to demonstrate relative protein amounts among samples. Percentage of Rho-GTP was calculated via Image J analysis [228].

Results

Retroviral shRNA eliminates Plxna1 expression in BMDCs

Previously, we used plasmid-based shRNA to target and reduce PlxnA1 expression in a DC-like cell line [71]. In the interest of examining a more biologically relevant system for the inhibition of PlxnA1 expression, we produced a retroviral-based RNA interference vector using the H1 promoter and a previously reported targeting sequence for generation of a short-hairpin structure that targets PlxnA1 mRNA (PlxnSh) for degradation in primary DCs (Fig. 2.1A) [71]. As a control, an identical PlxnA1 targeting sequence with a one-base pair mutation was cloned into the shRNA vector (CtrlSh). These vectors were used to generate viral particles for infection of DCs. GFP expression allowed us to determine that the virus was able to infect DCs with an efficiency of approximately 98% (not shown). Real-time PCR and immunoblotting indicate that DCs spinoculated with PlxnSh virus displayed greatly inhibited PlxnA1 mRNA and protein expression as compared to DCs infected with the mutated CtrlSh (Fig. 2.1B-C). Flow cytometry analysis of untransduced, CtrlSh and PlxnSh-transduced DCs indicates that expression of MHC-II (82.2%, 84.4%, and 85.7% positive, respectively) and co-stimulatory molecules (CD86: 97.3%, 98.4%, and 96.1% positive, respectively), were not altered by viral transduction or the absence of PlxnA1, reflecting specificity of the shRNA knockdown targeting (data not shown).

shRNA knockdown of PlexinA1 inhibits DC mediated T cell activation:

To functionally test the effect of retroviral PlxnSh in primary mouse DCs, we performed an antigen presentation assay using whole OVA protein or OVA peptide-pulsed

DCs and OT-II T cells. DCs transduced with PlxnSh virus exhibited approximately a >80% reduction in T cell stimulation compared with DCs transduced with CtrlSh as assessed by IL-2 production (Fig. 2.2A-B). Importantly, this reduction was observed with both whole OVA protein- and peptide-pulsed DCs (Fig. 2.2A-B). These observations suggest that the inhibition of T cell activation associated with PlxnA1 knockdown in DCs is not attributable to a defect in antigen processing or presentation by MHC class II.

Localization of plxnA1 in mature DCs at the Immune Synapse:

To examine PlxnA1 during DC maturation, DCs from C57BL6 mice were cultured in GM-CSF and IL-4 for 10 days and then in TNF-α for 2 additional days to achieve complete maturation [71]. Flow cytometry analysis of Day 10 and 12 DCs revealed $CD11c^+$ cells that lack expression of B220, CD14, and F4/80, indicating enrichment and uniformity of the population. Day 12 DCs exhibited a more mature phenotype demonstrated by higher expression levels of MHC class II when compared with their Day 10 counterparts (Fig. 2.3A). Immunofluorescent confocal microscopy was used to visualize the localization of PlxnA1 during DC maturation. Although PlxnA1 was absent in immature Day 6 DCs, it was detected in maturing Day 10 DCs as a cytoplasmic protein (Fig. 2.3B). Upon the addition of TNF-α, the protein became primarily located on the cell membrane and this pattern was sustained through Day 12 (Fig. 2.3B). As expected from previous reports, the MHC class II antigen (IA^b) was detected as an intracellular protein in immature DCs but localized to the membrane periphery as the DCs matured [231]. In Day 12 DCs, PlxnA1 was detected at the cell surface along with IA^b, and the merged image suggests colocalization (Fig. 2.3C).

Given PlxnA1's localization to the cell membrane in mature DCs, we examined whether PlxnA1 was found in the immune synapse. Mature day 12 DCs were pulsed with OVA overnight and incubated with T cells purified by negative selection column from splenocytes of OT-II TCR transgenic mice [232]. Staining DC-T cell conjugates for ICAM-1, IA^b, and TCR indicated that immune synapse-associated proteins of both DCs and T cells were enriched at the cell interface (Fig. 2.3). PlxnA1 localized to the DC-T cell interface in 70 ± 4.2% of DC-T cell conjugates (200 conjugates counted in five experiments). Analysis of IA^b, ICAM-1 and PlxnA1 staining demonstrates a non-punctate distribution at the interface that correlates with recent descriptions of the multifocal structures of the DC-T cell immune synapse not characterized by a *p*- or *c*-SMAC (Fig. 2.3D) [91]. TCR staining identified T cell conjugates while IA^b indicated DCs. ICAM-1, expressed by both T cells and DCs, is observed at the immune synapse.

PlxnA1 regulates actin polarization and Rho activation in DCs during T cell interactions:

Actin polarization in DCs occurs during their interaction with T cells, resulting in an accumulation of F-actin and the actin-bundling protein, fascin, at the DC-T cell interface [91, 233]. Given that Plxns are known to regulate actin and cytoskeleton rearrangements, we examined the possibility that PlxnA1 could regulate actin localization in DCs during interactions with T cells. Immunofluorescent staining of DC-T cell conjugates, revealed F-actin accumulation at the DC-T cell interface in WT DCs or CtrlSh DCs while DCs transduced with PlxnSh virus show dispersed actin (Fig. 2.4A). The F-actin signals are mostly attributed to DCs as nonassociated ad DC-associated T cells in these images do not exhibit much actin staining. Cells transduced with PlxnSh or CtrlSh were selected based on

GFP expression. The intensity of actin staining at the interface of DC-T cell conjugates was quantified (Fig. 2.4B). Additionally, a double-blind study revealed that cell conjugates formed wit PlxnA1-deficient DCs displayed reduced actin polarization in >50% of DCs when compared to controls (Fig. 2.4C).

Given that small GTPases are known to regulate the actin cytoskeleton, we next examined the ability of PlxnA1 to regulate Rho, Rac or Cdc42 in DCs during interactions with T cells [234]. OVA-pulsed PlxnSh- or CtrlSh-transduced DCs were incubated with fixed OT-II T cells for 20 min. prior to lysis. To analyze Rho activation from the DCs but not the T cells, OT-II T cells were fixed for 1 min. with 4% paraformaldehyde prior to incubation with DCs. Fixed OT-II T cells did not exhibit any significant accumulation of active Rho, Rac or Cdc42 (Fig. 2.4D, left panels). To assay for endogenous Rho, Rac and Cdc42 activity in DCs, the mixed cultures were lysed and precipitated with RBD or PBD-bound agarose beads for GTP-bound Rho, Rac or Cdc42 followed by immunoblotting (Fig. 2.4D, right panels, respectively). Lysate controls for total Rho, Rac or Cdc42 show equal loading on the SDS-PAGE gel. The percentage of GTP-Rho in relation to total Rho was calculated (Fig. 2.4D). The Rho family activation assay revealed two observations. First, efficient activation of Rho, Rac and Cdc42 in DCs requires antigen-specific interaction of T cells with DCs (compare pulsed to unpulsed DC). Second, inhibition of PlxnA1 expression by shRNA in DCs results in decreased levels of GTP-Rho compared to DCs transduced with CtrlSh virus (compare pulsed DC). Significantly, PlxnA1 inhibition affected Rho activation but not Rac or Cdc42 activation in pulsed DCs. These results illustrate that antigen-specific DC-T cell interactions are required for the activation of Rho, Rac and Cdc42 and that PlxnA1 regulates Rho activation in antigen presenting DCs.

Finally, we also examined whether a specific inhibitor of Rho activity would affect actin rearrangements in DCs conjugated with OT-II T cells. Immunofluorescent analysis illustrated that pretreatment of DCs with the Rho inhibitor, C3, greatly reduced the accumulation of F-actin in DCs at the interface with T cells (Fig. 2.4E). We also observed similar results with Y27632, an inhibitor of ROCK kinase (Rho kinase, an effector molecule of RhoA) (data not shown). Enumeration of cell conjugates demonstrated a >2-fold reduction in the percentage of DCs with synapse polarized actin in C3-treated samples vs control (Fig. 2.4F). These results clearly illustrate that Rho can regulate actin rearrangements at the immune synapse in DCs conjugated with T cells.

Discussion

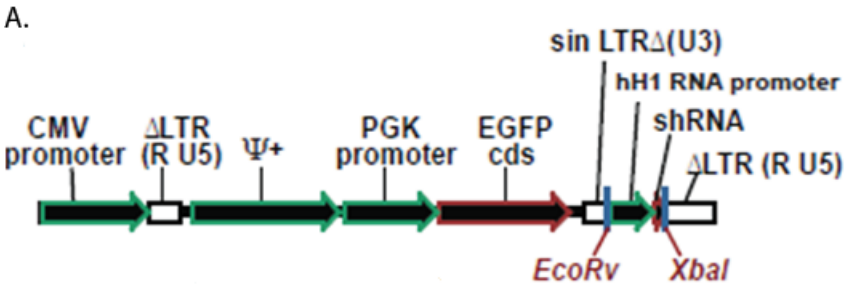
Through the exclusive use of primary DCs, we report that PlxnA1 expression is essential for optimal activation of T lymphocytes. PlxnA1 is expressed on the cell surface in a multifocal distribution that is characteristic of proteins in or near the immunological synapse between DCs and T cells (Fig. 2.3) [235]. We also report the novel finding of Rho activation and actin cytoskeleton regulation by a Plxn in the immune system. It is clear from our observations that PlxnA1 regulates Rho activation in DCs. Significantly, we observed that PlxnA1 regulation of Rho activation was distinct from activation of Rac or Cdc-42 (Fig. 2.4D). In the absence of PlxnA1 expression, we observed a significant loss of actin polarization, Rho activation, and T cell stimulation by DCs (Fig. 2.2 and 2.4). Previous studies have illustrated the importance of actin rearrangements at the immunological synapse for efficient T cell-DC interactions, and our data supports these findings. We suggest a model in which PlxnA1 regulates Rho activation and subsequent actin polarization in DCs. Our current work also demonstrates that PlxnA1 expression is critical for DC-mediated activation of T lymphocytes in a manner distinct from MHC class II processing or altered expression of co-stimulatory molecules (Fig. 2.2). One likely scenario is that Rho activation via PlxnA1 affects cell adhesion, dendrite formation, and thus, the ability of DCs to interact with multiple T cells. Supporting this model, we observed a significant reduction in actin polarization to the immune synapse in DCs treated with the Rho inhibitor C3 (Fig. 2.4E-F). In summary, this report begins to address the mechanism of DC-T cell regulation by PlxnA1 expression at the immune synapse.

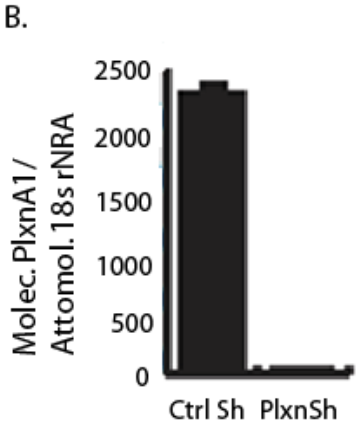
Acknowledgements

We thank Dr. Sergio Quezada and Dr. Steven Fiering for their help, and Dr. Michael Croft for the OT-II mice. We thank Dr. Neil Coffield and Dr. Lishan Su for the retroviral vector, pHSPG. We thank Dr. Keith Burridge and Lisa Sharek for their generous gift of RBD and PBD beads, Dr. Burridge and Dr. Channing Der for advice in the preparation of the manuscript, Dr. Heather Iocca for technical help with Image J analysis, and Dr. Robert C. Bagnell and Victoria J. Madden for their technical advice on confocal microscopy.

Figure 2.1. Retroviral shRNA to PlxnA1 inhibits expression. DNA cassettes consisting of the human H1 RNA promoter upstream of sequences encoding PlxnA1 or control mutated mouse shRNAs were prepared by DNA amplification using the mouse PlxnA1 shRNA vectors described previously (15) as template and the following oligonucleotide primers: 5'GCGATATCCCGCGGAATTCGAACGCTGAC-3' and 5'GCTCTAGAGGCCGGTATCGCTCGATT-3' (A). Transcription of the retroviral plasmid during viral production is driven by the immediate early enhancer/promoter region of the cytomegalovirus (CMV) promoter. Viral packaging is mediated by an extended packaging signal (Ψ^+) and long terminal repeat elements (ΔLTR (R U5)). A phosphoglycerate kinase (PGK) promoter drives expression of an enhanced green fluorescent protein (EGFP cds). Viral shRNA targeting of PlxnA1 eliminated the expression of PlxnA1 (B) mRNA and (C) protein (250kDa) in day 12 BMDCs. mRNA was assessed by realtime PCR, and the protein was assessed by immunoblotting (B & C).

Figure 2.1.


A.
sin LTRΔ(U3)
hH1 RNA promoter
shRNA
CMV promoter
ΔLTR (R U5)
Ψ+
PGK promoter
EGFP cds
ΔLTR (R U5)
EcoRv
XbaI


B.
Molec. PlxnA1 / Attomol. 18s rNRA
2500
2000
1500
1000
500
0
Ctrl Sh
PlxnSh

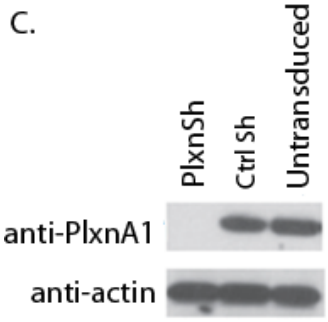

C.
PlxnSh
Ctrl Sh
Untransduced
anti-PlxnA1
anti-actin

Figure 2.2. shRNA interference of PlxnA1 inhibits DC mediated T cell activation. OVA peptide (323-339) (A) or whole OVA protein-pulsed DCs (B) transduced with PlxnSh or CtrlSh-containing virus were incubated with OT-II T cells. Lysates were assayed for IL-2 production by ELISA. Results are expressed as the mean of triplicate wells +/- SD from 3 experiments.

Figure 2.2.

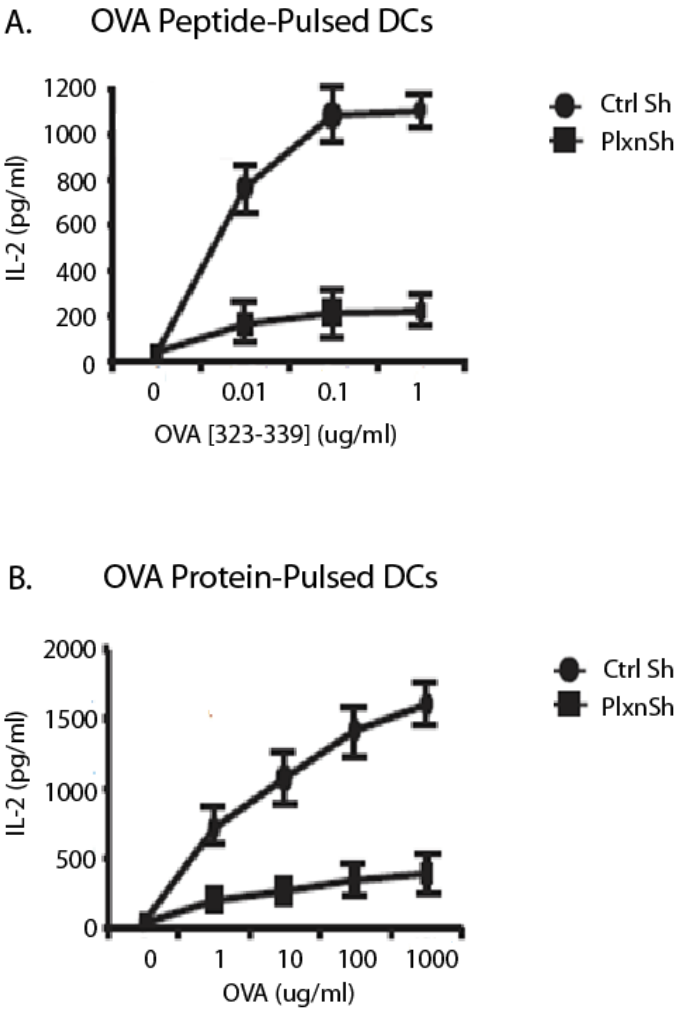

A. OVA Peptide-Pulsed DCs
IL-2 (pg/ml)
1200
1000
800
600
400
200
0
0
0.01
0.1
1
OVA [323-339] (ug/ml)
Ctrl Sh
PlxnSh
B. OVA Protein-Pulsed DCs
IL-2 (pg/ml)
2000
1500
1000
500
0
0
1
10
100
1000
OVA (ug/ml)
Ctrl Sh
PlxnSh

Figure 2.3. PlxnA1 localizes to the DC surface and the DC-T cell interface. Flow cytometry analysis of DC phenotype at days 10 and 12 of *in vitro* culture, comparing CD11c, MHC class II, CD8, B220, CD14 and F4/80 expression to an appropriate isotype control (A). DCs were collected on Day 6, 10 and 12 and stained for PlxnA1 (green) and IA^b (red) before resolving the images by confocal microscopy (B). Day 12 DCs were fixed and stained for PlxnA1 (green) and IA^b (red) (C). Matured bone marrow DCs were pulsed overnight with 50 μg/ml OVA and incubated with OT-II T cells for 45 min., fixed, permeabilized and stained for (top panels) TCR β (green), and IA^b (red), (second row) ICAM-1 (green) and IA^b (red), (third row) PlxnA1 (green), and IA^b (red) and (bottom panels) PlxnA1 (green), and ICAM-1 (red). PlxnA1 localized to the DC-T cell interface in 70 ± 4.2% of the 200 DC-T cell conjugates counted from five independent experiments (D). DIC, Differential interference contrast.

Figure 2.3.

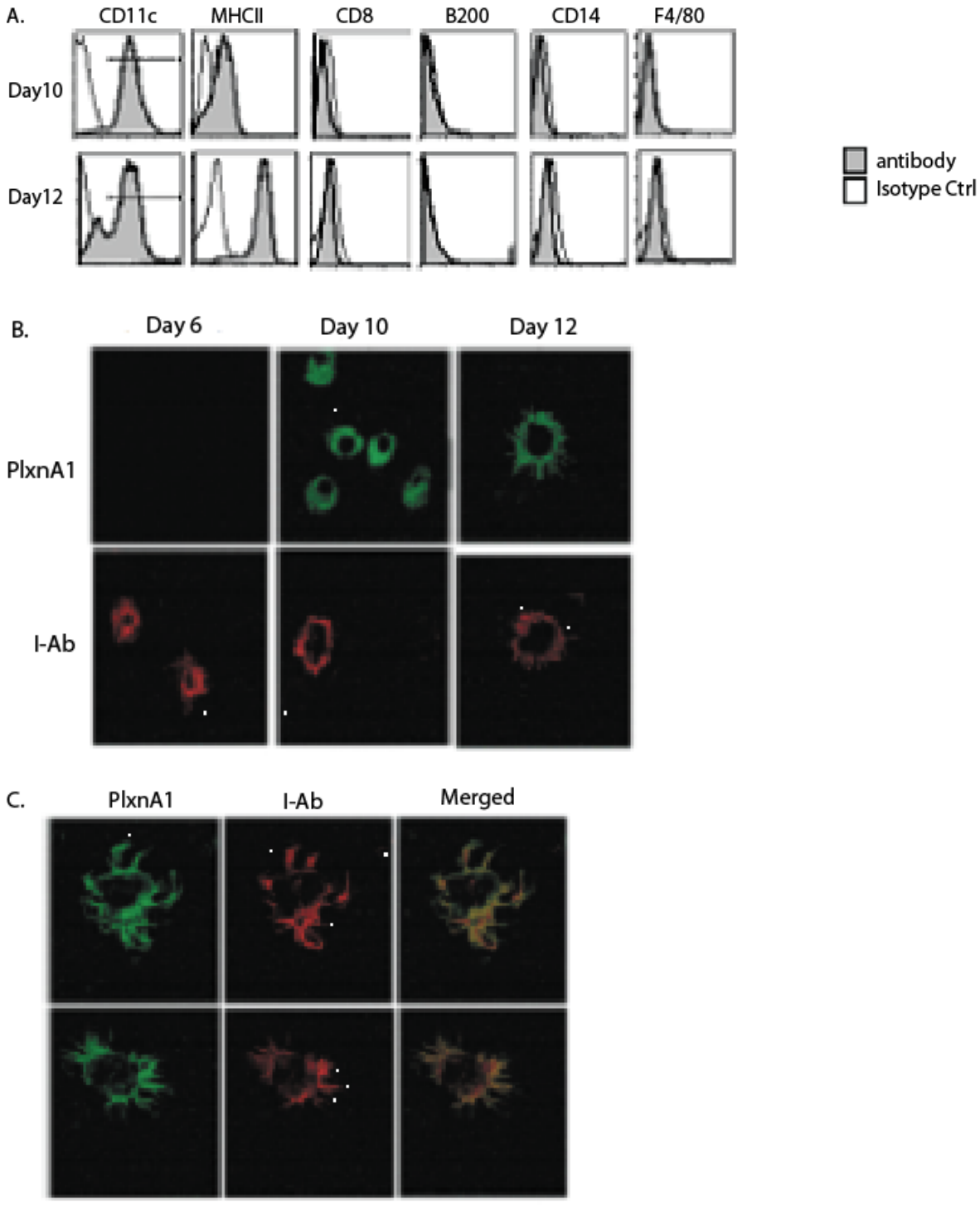

A.
CD11c
MHCII
CD8
B200
CD14
F4/80
Day10
Day12
antibody
Isotype Ctrl
B.
Day 6
Day 10
Day 12
PlxnA1
I-Ab
C.
PlxnA1
I-Ab
Merged

Figure 2.3.

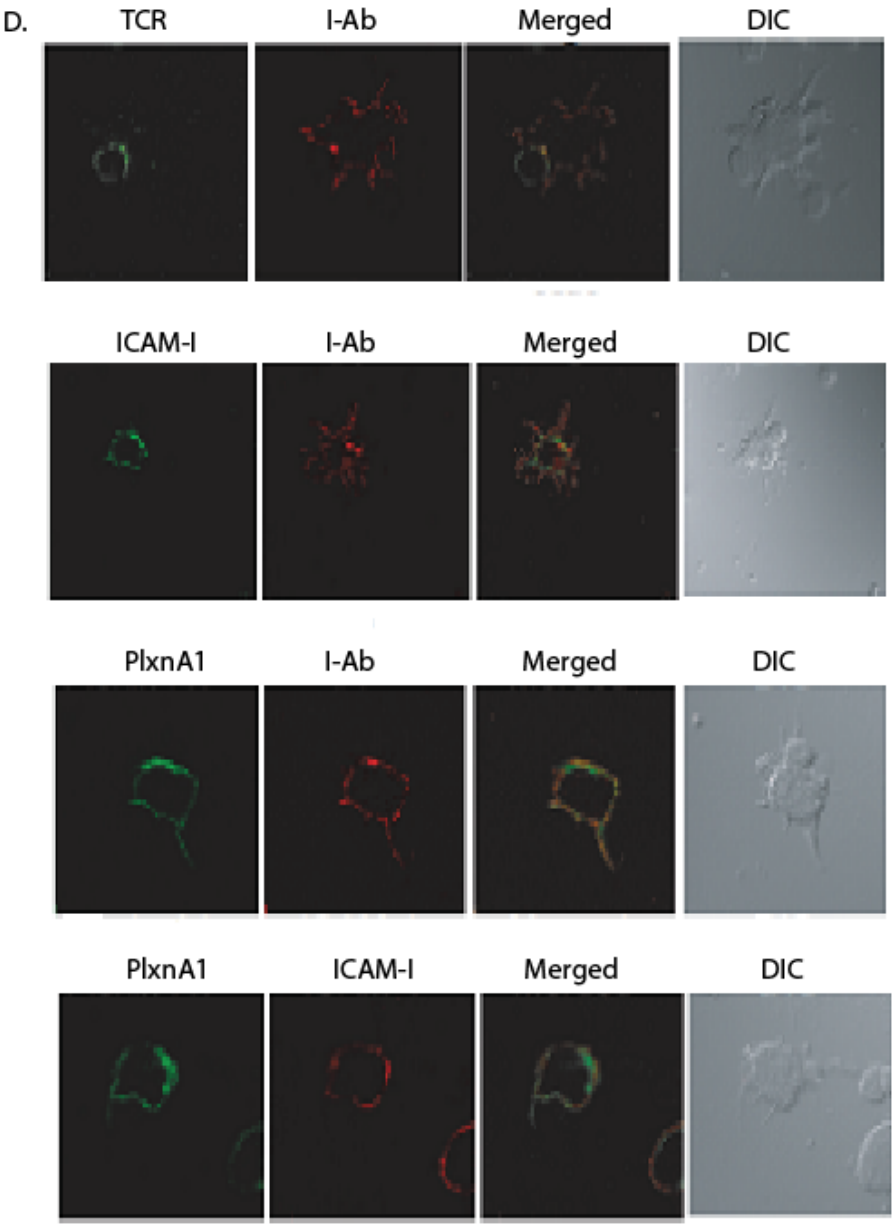

D.
TCR
I-Ab
Merged
DIC
ICAM-I
I-Ab
Merged
DIC
PlxnA1
I-Ab
Merged
DIC
PlxnA1
ICAM-I
Merged
DIC

Figure 2.4. PlxnA1 regulates polarization of actin at the DC-T cell interface. Conjugated DCs and T cells (1 x 10^5/cover slip) on poly-L-lysine-coated coverslips were fixed, permeabilized, and stained for actin and PlxnA1. Alexa 647-conjugated phalloidin was used to stain actin filaments. PlxnA1 in DCs was visualized via a polyclonal rabbit anti-mouse PlxnA1 antiserum, followed by incubation with an anti-rabbit IgG (H+L) Alexa 546-conjugated antibody. Analysis of PlxnA1 and actin expression was performed with WT DCs (top panels), PlxnSh DCs (middle panels), or CtrlSh DCs (bottom panels) as selected based on fluorescence of the viral GFP tag. Data are representative of six different experiments. T cells conjugated with DCs are highlighted with a white circle (A). Image J analysis was performed on multiple cell conjugates for actin fluorescence intensity at the DC-T cell interface (B). Actin-polarized DCs conjugated with T cells were counted in two independent double-blinded experiments and displayed as averages of percent polarized. A total of 70 cells for each sample were counted in each experiment (C). PlxnA1 regulates Rho activation but not activation of Rac1 or Cdc42. DCs transduced with PlxnSh or CtrlSh virus were pulsed with OVA and incubated with fixed OT-II T cells for 20 min. Cells were lysed and precipitated for GTP-Rho (top panels), GTP-Rac (middle panels), or GTP-Cdc42 (bottom panels), and immunoblotted with anti-Rho, anti-Rac, or anti-Cdc42 antibodies, respectively. Probing lysate controls for total Rho, Rac, or Cdc42 indicates approximately equal protein amounts. The percentage of GTP-Rho in relation to total Rho was calculated via Image J analysis. As a control, OT-II T cells at 5 x 10^6/100 μl were fixed (F) or unfixed (UF) prior to activation with anti-CD3ε, 145-2C11 (34) for 20 min., followed by a Rho, Rac, or Cdc42 assay. Blots are representative of three independent experiments (D). Immunofluorescent analysis of actin polarization in C3 treated DCs vs WT (E). Enumeration of the percentage of DCs with immune synapse polarized actin in C3-treated vs. untreated (WT) samples. A total of 70 cells were counted for each sample in each experiment (F).

Figure 2.4.

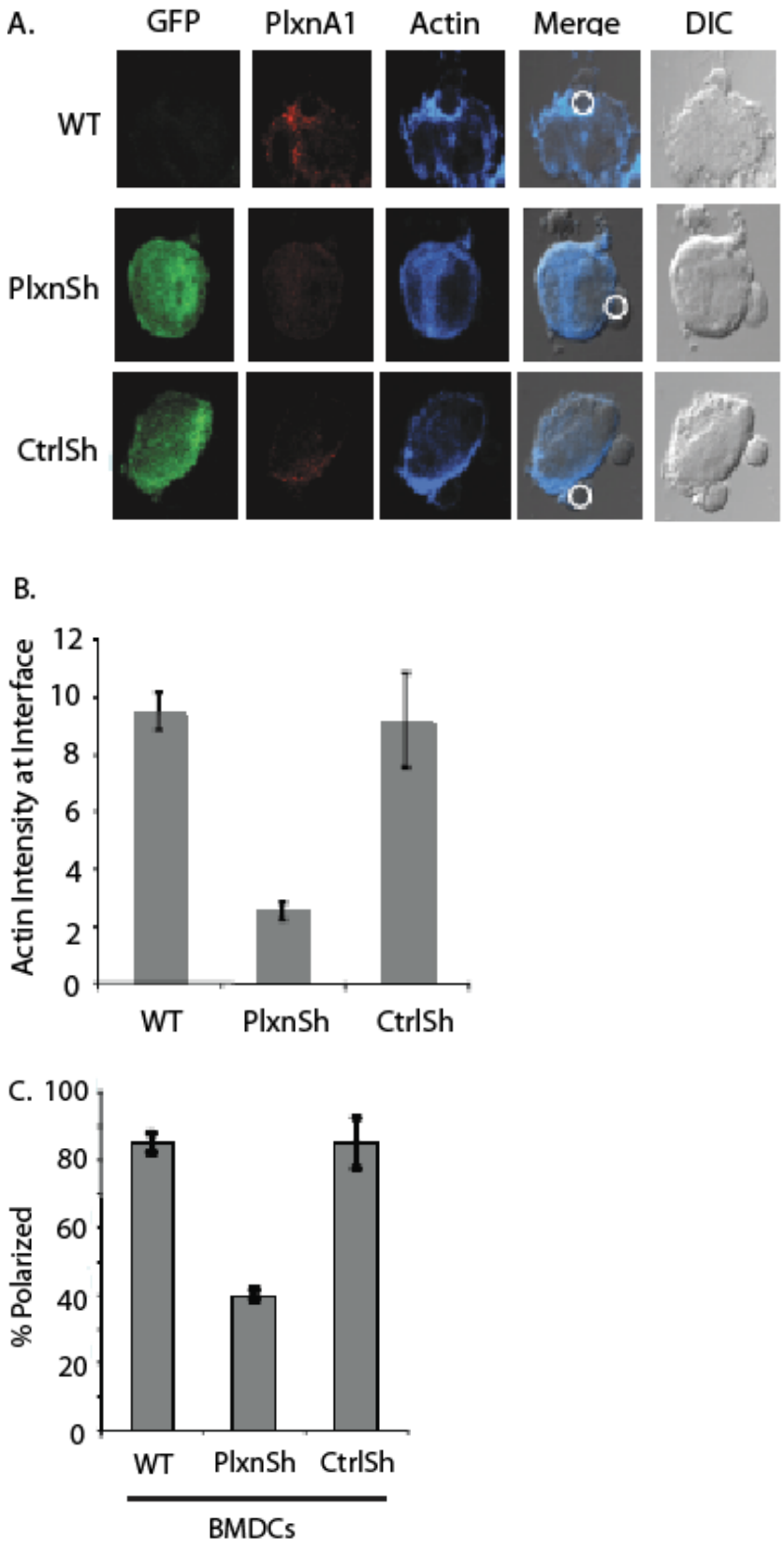
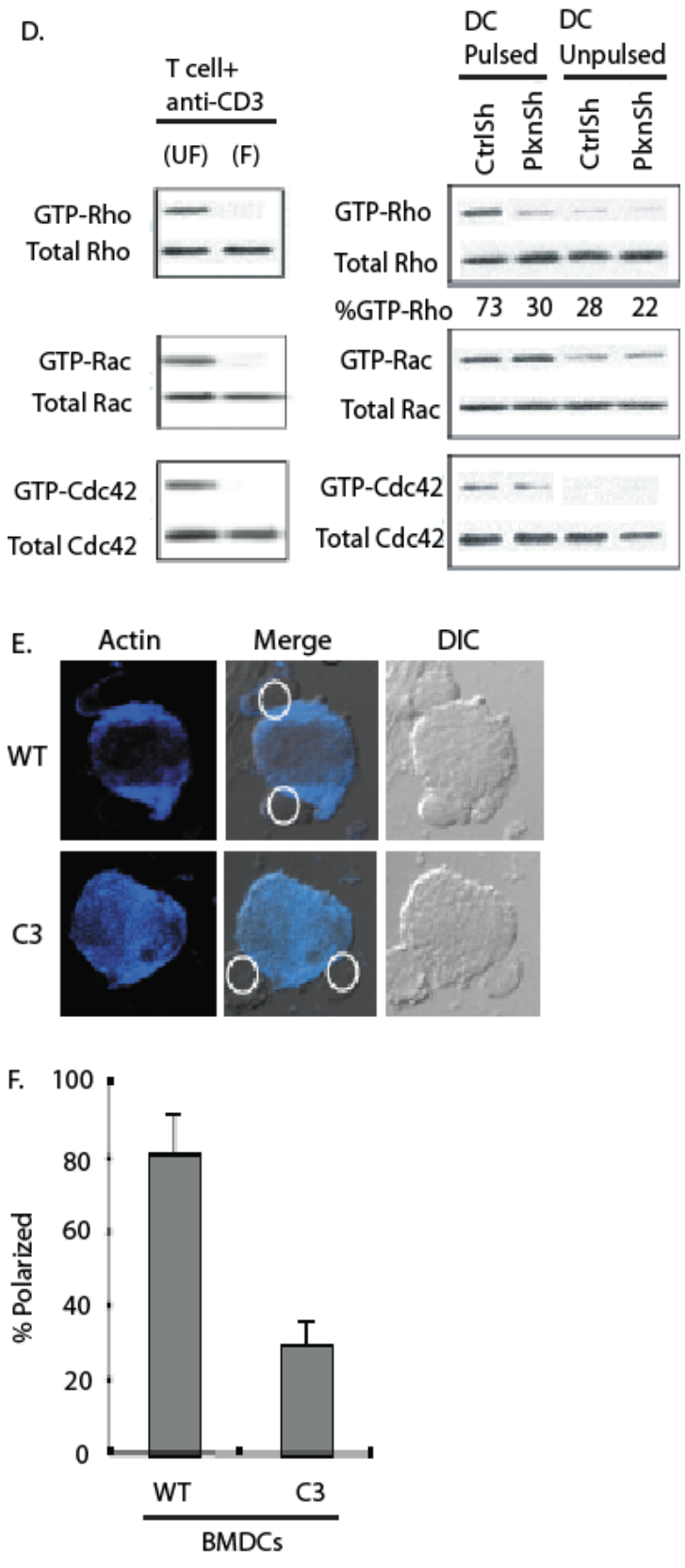

A.
GFP
PlxnA1
Actin
Merge
DIC
WT
PlxnSh
CtrlSh
B.
Actin Intensity at Interface
12
10
8
6
4
2
0
WT
PlxnSh
CtrlSh
C.
% Polarized
100
80
60
40
20
0
WT
PlxnSh
CtrlSh
BMDCs

Figure 2.4.


D.
T cell+
anti-CD3
(UF) (F)
GTP-Rho
Total Rho
GTP-Rac
Total Rac
GTP-Cdc42
Total Cdc42
DC
Pulsed
DC
Unpulsed
CtrlSh
PlxnSh
CtrlSh
PlxnSh
GTP-Rho
Total Rho
%GTP-Rho 73 30 28 22
GTP-Rac
Total Rac
GTP-Cdc42
Total Cdc42
E.
Actin
Merge
DIC
WT
C3
F.
100
80
60
40
20
0
% Polarized
WT
C3
BMDCs

CHAPTER III: A DUAL ROLE OF DENDRITIC CELL PLEXIN-A1 IN T CELL STIMULATION

Abstract

PlxnA1 was originally recognized as an axonal guidance molecule which is engaged by its ligand, semaphorin 3A, in neurons. Subsequently, PlxnA1 has been studied in a variety of systems, among which it stands out on mature dendritic cells (DCs), where it plays an important function in T cell priming. The current work shows that PlxnA1 plays a dual physiologic role on the surface of DCs by stimulating naïve T cell activation and by augmenting DC migration. Soluble PlxnA1-Fc fusion protein competitively inhibited antigen-dependent T cell activation stimulated by myeloid DCs expressing surface PlxnA1. In accordance, PlxnA1$^{-/-}$ DCs do not fully activate cognate T cells as demonstrated by co-culture of OVA-pulsed PlxnA1$^{-/-}$ DCs with T cells bearing OVA-specific TCRs. These experiments were extended to *in vivo* analysis, and the results show that PlxnA1$^{-/-}$ DCs have a reduced capacity to stimulate T cell activation resulting in severely reduced T cell expansion and significantly reduced numbers of interferon-γ expressing cells. Additionally, PlxnA1$^{-/-}$ DCs exhibit a reduced ability to migrate toward CCL19 or CCL21 *in vitro*. This result is recapitulated *in vivo* with PlxnA1$^{-/-}$ DCs exhibiting inefficient migratory activities to the draining lymph nodes based on the subcutaneous introduction of labeled DCs in mouse foot pads. Evidence provided by others suggests that Rap1 activity lies downstream of R-Ras, for which PlxnA1 is expected to exhibit GAP activity. Rap1 has also been shown to suppress Rho via Arap3 which performs a RhoGAP activity as a Rap1 effector protein. GST-pull-down assays were performed to capture active Rap1 in PlxnA1$^{-/-}$ or WT DCs conjugating with cognate T cells. Our preliminary data showed that Rap1 activity was elevated in PlxnA1$^{-/-}$ DCs, compared to WT controls upon T cell conjugation. Based on this result, the

presence of PlxnA1 correlates with reduced Rap1 levels as well as with subsequent activation of Rho in DCs, upon T cell contact. We hypothesize that suppression of Rap1 by PlxnA1 in line with R-Ras upon T cell conjugation not only signals for regulation of integrin activity but also for actin remodeling and polarization towards the T cell interface. We further hypothesize that PlxnA1 downregulates Rap1 to allow chemokine-induced DC migration, as well. Collectively, the current data indicate that PlxnA1 on DCs plays a dual role in regulating chemokine-induced DC migration and as a possible costimulator of T cells upon contact**.**

Introduction

Significance of PlxnA1 expressed on DCs: PlxnA1 was initially discovered in neurons, where it regulates axonal growth in response to semaphorin 3A (Sema3A), a soluble guidance factor [142]. Subsequent studies on PlxnA1 have been extended to various physiological contexts including cardiovascular development, and particularly immune responses fueled by our original findings that PlxnA1 expression is upregulated by CIITA (MHC Class II transactivator) during DC maturation [59, 66, 71, 236]. CIITA is the master regulator, controlling the expression of MHC class II molecules in antigen-presenting cells (APCs) [143-145]. Without the expression of MHC class II genes which is governed by CIITA, T cell-mediated adaptive immunity cannot be initiated, as exemplified by the human genetic disorder, Bare Lymphocyte Syndrome [146, 147]. PlxnA1 was found to constitute one of the genes regulated by CIITA [71]. Indeed, DCs that highly express PlxnA1 are the most potent APCs capable of activating naïve T lymphocytes, and initiating T cell-mediated adaptive immunity [148, 150-154].

To be able to prime T cells, DCs must accomplish several critical tasks. For priming T helper cells in particular, DCs must take up and properly process protein antigens for subsequent presentation of peptides on MHC class II molecules [148]. However, previous studies suggested that PlxnA1 is not involved in any of these functions. DCs lacking PlxnA1 do not show any defect in antigen uptake or in loading peptide antigens onto surface MHC Class II molecules [29, 71]. Furthermore, pulsing PlxnA1-depleted DCs with OVA peptides did not restore stimulatory T cell response, suggesting that antigen-processing is not the major step where PlxnA1 is involved in [71, 78]. PlxnA1 is also not critical for DC

upregulation of MHC class II and costimulatory molecules such as CD40, CD80 or CD86 necessary to steer T cells towards immunity rather than tolerance [29, 199, 200]. These results point to a role for PlxnA1 in the events that drive DC-T cell conjugation. This is supported by our own observation that actin polarization towards the DC-T cell interface, which is critical for T cell stimulation, is severely impaired in PlxnA1-depleted DCs, upon T cell engagement [71, 78, 91, 92].

Furthermore, we and others independently showed that, a T cell surface molecule, Sema6D can bind to PlxnA1 on DCs [29, 79]. Thus, PlxnA1 on DCs engages T cell surface molecules including Sema6D upon DC-T cell conjugation. A role of PlxnA1 in priming T cells upon contact could be verified by blockade with the competitive inhibitor, PlxnA1-Fc fusion protein which contains PlxnA1 ectodomain tethered to the Fc region of IgG. Analogous experiments using PlxnA1$^{-/-}$ DCs may confirm results with PlxnA1-Fc fusion protein blockade, and underscore the importance of this molecule in T cell proliferation.

Subpopulations of DCs: DCs are a highly heterogeneous cell type composed of different subsets of DCs. There are three major subtypes of DCs based on their differential surface markers: myeloid DCs (mDCs: CD11c^{+} CD11b^{+} CD8α^{-}); lymphoid DCs (lDCs: CD11c^{+} CD11b^{low} CD8α^{+}); and plasmacytoid DCs (pDCs: CD11c^{low} mPDCA-1^{+} B220^{+}), all of which are localized in the secondary lymphoid organs including the spleen [155-158].

The distinct nature of the ‘myeloid’ versus ‘lymphoid’ lineages of DCs has been under discussion with regard to their origin and function. It is still unclear whether CD8α^{-} mDCs and CD8α^{+} lDCs develop from distinct myeloid and lymphoid lineages [163-166]. Nontheless, their distinct pattern of distribution appears to comply with their

discrete functions: $CD8\alpha^+$ lDCs are thought to be tolerogenic, whereas $CD8\alpha^-$ mDCs are considered stimulatory [167]. Such stimulatory activity of $CD8\alpha^-$ mDCs seem to be supported by their superior phagocytic capacity [157, 168, 169]. However, both subsets of DCs have been observed to be stimulatory in other studies [170-174]. For example, both subsets of DCs have been demonstrated to equally induce antiviral CTL response *in vivo* [174]. In addition, it used to be assumed that $CD8\alpha^+$ lDCs were superior to $CD8\alpha^-$ mDCs in inducing Th1 response, due to the higher levels of IL-12p70 production by $CD8\alpha^+$ lDCs [173, 175-179]. That was because abundant IL-12p70 produced by lDCs was believed to be responsible for inducing IFN-γ, skewing Th1 response [176, 180, 181]. However, accumulated evidence suggests that DCs are not the only factors affecting T cell fates [182]. Moreover, recent publication revealed that there is a mechanism circumventing IL-12p70 for IFN-γ induction [183]. Thus, either subset of cells exhibit versatility by performing compensatory mechanisms, resulting in the induction of appropriate T cell responses initiated through different routes.

A third category of DCs, called pDCs express distinct markers such as mPDCA-1, Ly-6C as well as B220 [185-190]. pDCs can be induced to develop from mouse bone marrow cells *in vitro* by the addition of Flt3-Ligand [191, 192]. Functionally, pDCs are not well-equipped for antigen presentation, nor as potent as other subtypes of DCs in stimulating T cell-mediated immunity [185, 192-196]. pDCs also called IFN-producing cells are primarily devoted to the production of high levels of Type I IFN in response to viral challenge, inducing antiviral immunity [197]. Since a recent publication implicated PlxnA1 involvement in pDC function, it would be informative to profile PlxnA1

expression in these DC subtypes, in order to identify distinct roles for this molecule in these functionally diverse cell populations [30].

Migration of DCs: As mentioned previously, in order to achieve the unique capacity for T cell priming, DCs must undertake several critical steps prior to T cell engagement. One of those tasks is to migrate from the periphery to T cell areas in the secondary lymphoid tissues [237, 238]. CCL21 (SLC) and CCL19 (ELC) are the major chemokines that induce migration of mature DCs from the periphery to DLNs, by stimulating their receptor CCR7 on DCs [200, 239, 240]. Immature DCs express low levels of CCR7, which becomes upregulated upon DC maturation. CCR7-expressing DCs become mobilized upon stimulation with antigens or inflammatory cytokines such as TNF-α, and as a result, migrate into DLNs [208-210]. CCR7 on DCs is critical for DC migration, as $CCR7^{-/-}$ Langerhans cells in the skin are unable to migrate to DLNs [211]. Recent studies implicated CXCL12 (SDF-1α) as an additional chemokine important for DC migration, which binds to its receptor, CXCR4 on DCs [209, 241, 242]. Concurrently, other chemokine receptors such as CCR1 and CCR5, responsible for binding CCL5 (RANTES) are downregulated upon DC maturation [240].

PlxnA1 is a well-known pathfinder in neuronal axon growth in response to Sema3A, a soluble guidance cue, suggesting that PlxnA1 could also play an important role in chemokine-induced DC migration [4, 34, 140, 243]. Although no impairment was observed in DC migration to the DLNs in *plxna1*$^{-/-}$ animals by another group, their methodology which entails skin painting with fluorescent isothiocyanate (FITC) harbors complicating factors. It is imperative to reexamine the migratory capacity of DCs lacking PlxnA1, using hypothesis-driven experimental approaches [29].

Rho, R-Ras, and Rap Small G-proteins: Rho GTPase plays a role downstream of PlxnA1 upon T cell conjugation that directs actin polarization towards the T cell contact sites as shown in Chapter II [78]. Rho GTPase-mediated actin polarization in DCs has been shown to be critical for T cell activation [91, 244]. However, Rho GTPase (A, B or C) does not seem to directly interact with PlxnA1, leaving an unaddressed gap between PlxnA1 and Rho signaling [32, 33].

Functioanlly, PlxnA1 is anticipated to directly interact with R-Ras, a Ras-related small GTPase. PlxnA1 has been linked to regulation of R-Ras function through its potential GTPase activating protein (GAP) activity based on sequence homology [32, 105]. R-Ras is highly homologous to human Ras (H-Ras) [116]. Despite the sequence similarity, R-Ras has been demonstrated to play a distinct role from protooncogenic H-Ras [117, 118]. It is well established that R-Ras activates integrin signaling, resulting in cellular migration and adhesion [119]. A GAP is a regulatory element for a specific GTP-binding protein, suppressing G-protein signaling by facilitating G-protein-bound GTP hydrolysis. Upon facilitated hydrolysis of GTP, G-proteins become inactivated remaining bound to GDP [115]. Plxns contain segmented R-Ras GAP conserved regions including the invariant R residues critical for GAP activity [32, 105, 107]. In addition, circumstantial evidence suggests that PlxnA1 has GAP activity for R-Ras.

Rap1, a Ras-like small GTPase has been introduced as a downstream factor, activated by R-Ras [123]. Rap1 has also been implicated in translation of environmental stimuli into integrin-mediated cellular adhesion and motility, in general, and also in immune cells [124-132]. Rap1 has been shown to play a key role in chemokine-induced DC trafficking, through its effector molecule, RapL (Regulator of cell Adhesion and Polarization enriched in

Lymphoid tissues) [125]. Rap1 regulates not only integrin activity but also actin dynamics, through its diverse effector molecules [130, 245]. More importantly, Rap1 suppresses Rho activity by one of its effector molecules called Arap3, which exhibits GAP activity for Rho [246]. As mentioned previously in Chapter II, Rho is activated downstream of PlxnA1 upon T cell engagement [78, 246]. We are currently seeking to determine whether Rap1 activity is regulated by PlxnA1. R-Ras and Rap1 in DCs may be sequentially operational in the PlxnA1 pathway upon T cell engagement. However, it is also possible that PlxnA1 directly suppresses Rap1 activity without involving R-Ras, since PlxnA1 contains a SynGAP conserved region which is known to be essential for the GAP activity of Rap [32, 136]. The latter possibility is also supported by the findings that Rap1 and R-Ras share some of their GAP proteins [137]. Our primary question is whether Rap1 activity is regulated by PlxnA1 and if that results in Rho activation upon T cell engagement.

Materials and Methods

Medium: Cells were cultured in complete RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (ATLANTA Biologicals), 2.0 mM L-Glutamine, 50 µM β-mercaptoethanol, 25mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, and sodium pyruvate, as previously described [184].

Cells and mice: Bone marrow-derived DC (BMDCs) were generated as previously described [71, 78, 184]. Briefly, DCs were enriched from mouse bone marrow cells (2 x 10^5/ml) for 12 days by the addition of GM-CSF (Day 0: 20 ng/ml; Day 3: 10 ng/ml; Day 6: 10 ng/ml; Day 8 and 10: 5 ng/ml) and IL-4 (Day 6 and 8: 10 ng/ml; Day 10: 5 ng/ml), as well as TNF-α (Day 10: 20 ng/ml; Day 11: 10 ng/ml) (Peprotech) in complete RPMI-1640 medium. Cells were harvested at Day 12 and used for *in vitro* migration assays or further pulsed with 1 µM ovalbumin (OVA) protein (Worthington Biochemical) or 0.1 µM OVA peptide (323-339, GenScript) at 37^{o}C, overnight. OVA-pulsed DCs were used for co-culture experiments or for injection into recipient mice along with CFSE-labeled OT-II T cells that have been enriched using Mouse T Cell Enrichment Columns or Mouse T cell CD4 Subset Columns (R&D Systems). For real-time PCR analyses of PlxnA1 expression, the splenocytes or the bone marrow cells isolated from C57BL/6 mice were sorted using MoFlo (Dako), following staining with Phycoerythrin (PE)-, Fluorescein isothiocyanate (FITC)- or Allophycocyanin (APC)-conjugated antibodies against cell surface markers: CD19 (B cells), CD3 (T cells), CD11c and CD11b (mDCs and macrophages (Mac)) and CD11c, B220 and mPDCA-1 (pDCs). (eBioscience). BM-derived pDCs were enriched with Flt3L (R&D

Systems) treatment for 10 days in complete RPMI 1640 medium at 37°C in 5% CO_2, as described [191], and the mPDCA-1^+ $B220^+$ and $CD11c^+$ pDCs isolated by FACS. C57BL/6 mice were obtained from Jackson Laboratory. The C57BL/6 Ly5.1, recipient mice used for adoptive transfer experiments were purchased from the National Cancer Institute, Bethesda, MD. *Plxna1*$^{-/-}$ mice were provided by our collaborators, Dr. Yutaka Yoshida and Dr. Thomas Jessell at Columbia University [243]. The *plxna1*$^{-/-}$ mice back-crossed for five generations into the C57BL/6 strain at Columbia University were backcrossed an additional four generations into the identical strain. OT-II-C57BL/6 mice which are $OVA_{323-339}$ peptide-specific TCR transgenic animals were obtained from Dr. Michael Croft (La Jolla Institute for Allergy and Immunology, La Jolla, CA) [78]. Animals were housed at UNC-Chapel Hill under specific pathogen-free conditions, and animal procedures were performed in compliance with the National Institutes of Health *Guides for the Care and Use of Laboratory Animals*, as pre-approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Preparation of cDNA and Real-Time quantitative PCR: Total RNA was isolated from each population of freshly isolated or *ex vivo* cultured cells, using RNeasy Plus total RNA isolation kit (Qiagen), from which complementary DNA was generated using random primers and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturers' instructions. Real-time PCR was performed using CyBrGreen Mix (Applied Biosystems) and the following oligonucleotides [79]: *Plxna1* primers, forward 5'-CAATCCTGCTACCGTGGAGAA-3', reverse 5'-CCGCAGAAGTCGTCATCAAT-3' [71]; *Ccr7* primers, forward 5'-AAAGCACAGCCTTCCTGTGT-3', reverse 5'-

AGTCCACCGTGGTATTCTCG-3' [247]; *Cxcr4* primers, forward 5'-CAGAGGCCAAGGAAACTGCT-3', reverse 5'-CTGACGTCGGCAAAGATGAA-3' [248], and *β-actin*, forward 5'-AGGGCTATGCTCTCCCTCAC-3'; reverse 5'-CTCTCAGC TGTGGTGGTGAA-3' [79, 248]. Real-time PCR was performed using AB Prism 7700 instrument (Applied Biosystems). The levels of *Plxna1, Ccr7, and Cxcr4* transcripts were normalized to *β-actin* expression [79]. Each experiment had triplicate samples and each value shown is representative of three independent experiments.

Cloning and expression of PlxnA1-Fc: The extracellular fragment (265-3969 bp) of full-length Plxna1 cDNA sequence was subcloned in TOPO-TA (Invitrogen), and fused to the 5'-end of the Fc portion of human IgG1 cDNA (792-1487 bp: Hinge-CH2-CH3) in the pCEP4 mammalian expression vector (Invitrogen). A Kozak sequence (GCCACCC) was added at the 5'-end of PlxnA1 upstream of the translation start site (ATG, 265-267 bp) followed by its intrinsic signal peptide sequence. For a Fc-only construct, Kozak, ATG and IL-2 signal sequences (TAC AGG ATG CAA CTC CTG TCT TGC ATT GCA CTA AGT CTT GCA CTT GTC ACG) were added to the 5'-end of the Fc portion of human IgG1 (792-1487) along with restriction enzyme sites at either end using PCR, subcloned into TOPO-TA and cloned into the pCEP4 backbone (Invitrogen). Both the constructs were sequenced, transiently over-expressed in HEK293 cells, and the resulting protein purified using protein A/G agarose beads (Pierce). Transient expression was established by introducing the pCEP4 cDNA construct of PlxnA1-Fc or Fc-only into HEK293 cells using calcium-phosphate co-precipitation [249]. After a 72 hr-incubation in serum-free IMDM medium (Gibco), culture supernatant was removed from the cells, and passed through sterilizing-grade filters

(Millipore). Prior to the purification step, expression of PlxnA1-Fc and Fc-only control protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting. Briefly, filtered supernatants were incubated with Protein A/G agarose beads at 4^{o}C overnight. Beads were washed three times with PBS, and boiled in SDS-PAGE loading buffer. Protein eluted from the beads was subjected to 8% or 15% SDS-PAGE followed by Coomassie blue staining or transfer to nitrocellulose membrane. PlxnA1-Fc or Fc-only protein was detected using the anti-Fc of the human IgG conjugated to horse radish peroxidase (Invitrogen). For functional assays, PlxnA1-Fc and Fc-only protein were purified using Protein A/G columns (Invitrogen), dialyzed with PBS, and concentrated using Amicon Ultra (100 kDa or 10 kDa, Millipore). Protein concentration was determined by Bradford protein assay, and the samples stored in 25% glycerol/PBS at -70^{o}C.

Co-Culture of DCs with OT-II T cells: Prior to DC-T cell co-culture, BMDCs from *plxna1*$^{-/-}$ mice and WT controls were examined for the expression of the following surface markers using either FITC-conjugated or PE-conjugated fluorescent antibodies as followed: anti-CD86-PE, anti-CD80-PE, anti-I-Aβ-FITC and anti-CD40-PE along with anti-CD11c-APC (BD Biosciences). BMDCs (2 x 10^{5}) were co-cultured with 1 x 10^{6} OT-II T cells in complete RPMI-1640 medium. Prior to co-culture, CD3^{+} T cells or CD4^{+} T cells were enriched employing Mouse T cell enrichment columns or Mouse T cell CD4 Subset Columns (R&D Systems), respectively, according to the Manufacturer's instructions. Cells were labeled with Carboxy-Fluorescein Succinimidyl Ester (CFSE: Sigma) by incubation in serum-free RPMI medium with 1 μM of CFSE at 37^{o}C for 10 min. followed by several washes with complete RPMI medium [79]. For blocking experiments, PlxnA1-Fc or Fc-only

control protein was added to the DC-T cell co-culture at Day 0. Cultured cells were harvested at specific time points post co-culture. Harvested cells were treated with anti-mouse CD32/CD16 Fc blocker, and stained with APC-conjugated anti-TCR antibody (anti-TCR-APC) or with biotinylated anti-Vβ5 antibody or anti-CD69 (anti-Vβ5-biotin or anti-CD69-biotin) followed by streptavidin conjugated with APC (SA-APC) (eBioscience). Cells were fixed in 1% formaldehyde/PBS and stored at 4^{o}C for flow cytometry. Cells from each time course were analyzed at the same time by flow cytometry using the Dako Cyan cytometer and analytical software, Summit 4.3 or FlowJo (TreeStar).

Intravenous Co-Transfer of DCs and CFSE-labeled OT-II T cells: CFSE-labeled OT-II T cells (2 x 10^6) and OVA-pulsed BMDCs (5 x 10^5) from *plxna1*$^{-/-}$ mice or WT control mice were simultaneously injected intravenously into the tail vein of C57BL/6 Ly5.1 mice. Splenic T cells isolated from the spleen with hypotonic RBC-lysis (15.5 mM NH_4Cl; 1 mM $KHCO_3$; 10 μM EDTA in H_2O) 72 hr. post-transfer were analyzed. Three mice were used per group in each experiment and the data shown is representative of two independent experiments

Analysis of OT-II T cells from recipient mice: The spleen from each recipient mouse that was co-injected intravenously with DCs and CFSE-labeled OT-II T cells was isolated and prepared as a single cell suspension following erythrocyte depletion with hypotonic RBC-lysis. Splenocytes were treated with anti-mouse CD32/CD16 Fc blocker and stained with anti-Vβ5-biotin followed by SA-Pacific Blue (PB), along with anti-Ly5.2-APC-Cy7 and anti-TCR-APC (eBioscience). The Vβ5$^+$ cell population gated out of the viable Ly5.2$^+$ TCR$^+$ T

cell population was analyzed for the presence of a CFSE fluorescence shift using flow cytometry.

ELISPOT assay: IFN-γ ELISPOT assays were performed according to the manufacturer's instructions (eBioscience). Briefly, a 96 well ELISPOT plate was pre-coated with anti-IFN-γ antibody at 4^{o}C overnight. Splenic single cell suspensions were prepared as described, and re-stimulated with peptide $OVA_{323-339}$ (0.1 µM) or left unstimulated in complete RPMI medium for 24 hr. at 37^{o}C with 5% CO_2. The cells were serially diluted 1:1 to 1.25 x 10^5 cells, starting from 2 x 10^6 cells. After a 24 hr-incubation, cells were removed by washing and IFN-γ detected using biotinylated anti-IFN-γ antibody followed by Avidin-Horse Radish Peroxidase (Av-HRP). Freshly prepared AEC (3-amino-9-ethyl carbazole) substrate solution supplemented with 0.015% H_2O_2 was added to each well, and developed for 10 to 45 min. The reaction was stopped with distilled water, and the plates dried overnight in the absence of light. Purple spots, each of which representing a cell that had secreted IFN-γ, were enumerated using a microscope. Spots were counted double blinded, and the differences between the two groups (w/ WT DCs vs w/ PlxnA1$^{-/-}$ DCs) were evaluated by a student t-test.

***In vitro* Migration Assay**: Ninety six trans-well Chemo Tx plates (5 µm pore size; NeuroProbe) were used as previously described [184]. Briefly, the chemokines, CCL19, CCL21, CXCL12, and CCL5 (PeproTech) as well as the complement factor, C5a (R&D Systems) were serially diluted at a 1:1 ratio in 28 µl of serum-free RPMI 1640 medium and placed in the bottom wells. BMDCs (1 x 10^5) from *plxna1*$^{-/-}$ mice or WT controls suspended in serum-free RPMI-1640 medium were added onto the hydrophobic membrane filter, and

the assembly incubated for 3 hr. at 37^{o}C in a 5% CO_2 incubator. Cells on the filter were removed by aspiration, and the cells still remaining on the filter washed with Versene and PBS. Cells that migrated through the filter were spun down at 1,500 rpm at 4^{o}C for 5 min. Medium from each bottom well was transferred to the corresponding well of a 96 well ELISA plate (Corning) for a XTT assay.

XTT Assay: XTT viability assays were performed on the cells present in the bottom wells of the trans-well plates. XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] (Sigma-Aldrich) was dissolved in pre-warmed serum-free RPMI 1640 medium at 1 mg/ml. PMS (Phenazine MethoSulfate: Sigma-Aldrich) was dissolved at 1.5 mg/ml in PBS and stored at 4^{o}C for up to 3 hr. Fifty micro-liters of PMS solution were added to 10 ml XTT solution, 50 µl of which was then added to each of the 96 wells. The plates were incubated for 4 hr. at 37^{o}C in the presence of 5% CO_2. A standard reference plate was prepared using 1 x 10^5 cells serially diluted at a 1:1 ratio. The number of cells that migrated was deduced from the standard curve made from the standard reference plates. Optical density was measured at 450 - 650 nm using a spectrophotometer, Spectra Max 190 (Molecular Devices). The average of triplicate wells reflecting one concentration of each chemokine was displayed with standard deviations. All the experiments were performed in triplicate.

Subcutaneous Transfer of BMDCs into Mouse Foot Pads: BMDCs (2 x 10^6) generated from *plxna1*$^{-/-}$ mice and from WT control mice were simultaneously injected subcutaneously into mouse foot pads following differential labeling with CMFDA (CellTracker^{TM} Green

CMFDA: 5-chloromethyl fluorescein diacetate; Molecular Probes) on PlxnA1$^{-/-}$ DCs and CMRA (CellTracker^{TM} Orange CMRA: Molecular Probes) on WT DCs. An identical was performed with the only variation being the switching of the labeling dyes. Labeling of the cells with either reagent was performed according to the manufacturer's instructions. Briefly, a final concentration of 0.8 µM CMFDA or CMRA was added to 1 x 10^7/ml of BMDCs derived from *plxna1*$^{-/-}$ or WT mice in pre-warmed PBS (Mg^{2+}- and Ca^{2+}-free) and incubated for 30 min. at 37^{o}C. After washing twice with PBS, cells were incubated in PBS for another 30 min. at 37^{o}C. Differentially labeled cells were mixed in equal ratios (CMRA$^+$ WT mixed with CMFDA$^+$ KO; CMFDA$^+$ WT mixed with CMRA$^+$ KO) and prepared at 2 x 10^6 cells /30 µl of PBS for subcutaneous transfer into the foot pads of the recipient C57BL/6Ly5.1 congenic mouse. Thirty six hours post-BMDC transfer, the recipient mice were euthanized and their popliteal draining lymph nodes (DLNs) isolated along with the non-DLNs (axillary). Both sides of the popliteal LNs were pooled together per mouse, and single cell suspensions prepared in 2% FCS-containing PBS by gently grinding the tissues with pellet pestles and filtering the suspension through a cell strainer (BD Biosciences). Cells (1 x 10^6) were treated with anti-CD32/CD16 Fc blocker and then stained with anti-CD11c-APC-Cy7 along with anti-Ly5.2-APC. The cells gated out of viable Ly5.2$^+$ populations were analyzed in two-dimensional plots with CMRA (FL2) and CMFDA (FL1), by flow cytometry (Flow Cytometer, Dako Cyan). Four to eight recipient mice were used per group, resulting in a total 16 to 32 mice per experiment. The result shown is representative of three independent experiments. Statistical analysis between the two groups per set was carried out using a student t test (Prism Software).

GST pull-down assay for activated Rap1: DCs (1×10^6) were conjugated with OT-II T cells (1×10^6) that had been fixed with 1% formaldehyde at 37°C for 2 min., as described previously [78, 246, 250]. Cells were lyzed in a modified immune precipitation assay buffer (50 mM Tris, pH 7.4, 75 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), containing protease inhibitors as well as 1 mM NaF, and 1 mM sodium vanadate. Cell debris was removed by centrifugation and lysates incubated with 50 μg of GST-RalGDS-RBD (provided by Dr. Leslie Parise, University of North Carolina at Chapel Hill) immobilized on glutathione beads to capture GTP-bound active Rap1. The amount of active Rap1 and total Rap1 from the lysate was determined by Western blotting with a polyclonal anti-Rap1 antibody (Santa Cruz Biotechnology) that recognizes both Rap1A and Rap1B, followed by an anti-rabbit IgG HRP secondary antibody (Santa Cruz Biotechnology). The relative amount of active Rap1 was determined by comparing the amount of pulled-down active Rap1 to the total amount of Rap1 from cell lysates.

Results

PlxnA1 is primarily expressed in myeloid DCs.

DCs are comprised of highly heterogenous cell types, including subsets of which exhibit overlapping but distinct properties and functionalities. Although PlxnA1 was originally found to be highly expressed in mature BMDCs, we profiled PlxnA1 expression patterns in different subsets of DCs along with other immune cells. PlxnA1 transcript levels in different subsets of freshly isolated DCs and bone marrow-derived DCs, along with other cell types such as B and T lymphocytes were determined by real-time quantitative PCR. PlxnA1$^{-/-}$ BMDCs were used as a negative control for PlxnA1 expression (Fig. 3.1). PlxnA1 was found to be predominantly expressed in DCs, with the highest expression detected in mature BMDCs cultured for 12 days *in vitro*, followed by freshly isolated splenic (CD11c^{+} CD8α^{-}) mDCs and the mDCs isolated from bone marrow that were not subjected to culture (Fig. 3.1). Splenic (CD11c^{+} CD8α^{+}) lymphoid DCs (IDCs) exhibited PlxnA1 expression but the level was lower than their myeloid counterparts from the spleen [155-158]. In addition, it is notable that mature BMDCs treated with TNF-α showed higher levels of PlxnA1 expression than those of freshly isolated DCs with no stimulation, confirming our observations that PlxnA1 is upregulated upon maturation [71]. PlxnA1 transcripts were also detected in BM-derived macrophages (Mac), but not in unstimulated peritoneal cells mainly composed of Macs remaining in a resting state (Fig. 3.1). PlxnA1 mRNA levels were maintained very low in freshly isolated splenic B and T lymphocytes [155-158]. In addition, either freshly isolated or *in vitro* Flt3L-induced (CD11c^{+} mPDCA-1^{+} B220^{+}) pDCs from bone marrow did not express significant levels of of PlxnA1. This is contrast to a previous

report which demonstrated PlxnA1 transcription in pDCs, where it was suggested to be involved in TLR signaling [30]. Nevertheless, PlxnA1 appears to be expressed at much higher levels in myeloid lineages, especially in BMDCs.

PlxnA1-Fc inhibits T cell proliferation.

Accrued evidence suggests a role for PlxnA1 is mainly in DC-T cell conjugation. PlxnA1 is primarily involved in actin polarization upon T cell engagement as previously addressed in Chapter II [78]. In addition, one of the ligands for PlxnA1, Sema6D, is expressed on T cells [29, 79].

To directly test whether PlxnA1 plays a role upon T cell contact, soluble PlxnA1-Fc fusion protein was utilized, as a competitive inhibitor of PlxnA1. PlxnA1-Fc fusion protein is composed of the PlxnA1 extracellular region linked to the Fc portion of the human IgG1 molecule resulting in it being secreted as a soluble protein (Fig. 3.2A). The truncated PlxnA1-Fc protein is expected to associate with PlxnA1 ligands expressed on T cells, by competing with endogenous PlxnA1 on DCs. As a control, the Fc portion of human IgG1 alone was generated. The Fc-only protein was designed to be secreted by the addition of a signal peptide sequence to the 5'-end of the Fc portion (Fig. 3.2A). The soluble PlxnA1-Fc and Fc-only coding sequence was cloned into the pCEP4 mammalian expression vector, and the protein products generated in HEK293 cells. Both of the proteins were tested for purity as well as the level of expression using SDS-PAGE and Western blotting. As shown on a SDS-PAGE gel visualized by Coomassie blue, the molecular weight of PlxnA1-Fc and Fc-only was approximately 180 kDa, and 25 kDa, respectively, as predicted (Fig. 3.2Ba). This

was confirmed by Western blotting with HRP-conjugated anti-human Fc IgG (Fig. 3.2Bb). Both of the proteins produced in HEK293 in serum-free condition were highly enriched with little contamination (Fig. 3.2Ba-b). Their expression levels were also relatively high, compared to that of the same coding sequence cloned in other vectors (data not shown).

For a DC-T cell co-culture experiment, freshly isolated splenic OT-II T cells were labeled with CFSE, and put into culture along with OVA protein-pulsed BMDCs in the presence of PlxnA1-Fc protein or Fc-only protein. As a negative control, DCs left unpulsed (No OVA control DCs) were used in cultures with T cells. Cells were harvested at 96 hr. post co-culture, and stained with Vβ5-biotin followed by APC-streptavidin. The cells gated as the $V\beta5^{+}$ live lymphocyte population was analyzed according to its CFSE peaks. The PlxnA1-Fc-treated group showed less efficient T cell proliferation than the Fc-only group. As shown in Figure 3.2C, proliferating cells accumulated more in the second peak (39.6%), representing the first descendants of the originally CFSE-labeled ancestors, while only 27.6% of the cells in the second peak were detected in cells treated with Fc-only protein. In contrast, the proportion of dividing cells that have undergone further division was dramatically reduced in the PlxnA1-Fc treated group, 15,6%, 4.4% and 1.8% in the 3rd-5th peak, respectively (Fig. 3.2C, the middle panel). This is reduced when compared to control cells treated with Fc-only which have 20.8%, 8.5%, and 5.4% of cells in the 3rd-5th peaks respectively (Fig. 3.2C, the left panel). The OT-II T cells stimulated by DCs without OVA pulse did not proliferate, as expected (Fig. 3.2C, the right). These results support the contention that PlxnA1 participates in stimulating T cells at the level of cell-cell contact, in line with antigen-loaded MHC class II and co-stimulators including B7 molecules, in an antigen-dependent manner.

Data in Figure 3.2 are from a co-culture representative of three independent experiments using the $CD3^+$ T cells from OT-II transgenic mice. Experiments using $CD4^+$ OT-II T cells also show similar reduced proliferation patterns in the PlxnA1-Fc treated group compared to Fc-only controls (data not shown). Regarding the stimulatory effect of PlxnA1-Fc on T cells through its binding to Sema6D or other ligands on T cells, the addition of PlxnA1-Fc into a co-culture of OT-II T cells and $PlxnA1^{-/-}$ DCs did not improve proliferation efficiency compared to those with Fc-only protein, suggesting that PlxnA1-Fc has little effect on T cell proliferation. This is further supported by the lack of augmentation effect of PlxnA1-Fc in T cell proliferation upon anti-CD3 and ani-CD28 stimulation (data not shown).

$PlxnA1^{-/-}$ DCs are less effective in stimulating co-cultured OT-II T cells.

DC-T cell co-culture with PlxnA1-Fc demonstrated that T cell proliferation is inhibited upon interfering with the PlxnA1 interaction with its ligand on T cells (Fig. 3.2C). However, the inhibition by PlxnA1-Fc might have been partial, due to its competitive binding with endogenous PlxnA1 from DCs. Therefore, the blocking effect of PlxnA1 should be further examined in the future by utilizing different dilutions of PlxnA1-Fc and Fc-only to determine an optimal blocking concentration. Considering the possibility of partial inhibition by PlxnA1-Fc, DCs completely lacking PlxnA1 ($PlxnA1^{-/-}$ DCs) could be used instead. T cell-stimulation with $PlxnA1^{-/-}$ DCs would clearly show the severity of the defect in T cell response caused by lack of PlxnA1 on DCs.

Prior to co-culture experiments, it was confirmed that the expression of co-stimulatory molecules such as CD86, CD80, or CD40 along with CD11c is not affected by the removal of PlxnA1, and neither is that of $I\text{-}A^b$, as previously determined (Fig. 3.3A) [29, 71]. As

suggested by our studies using RNAi or PlxnA1-Fc protein (Fig. 3.2), our co-culture experiments using PlxnA1$^{-/-}$ DCs revealed that T cells bearing OVA$_{323-339}$-specific TCRs were not activated in the absence of PlxnA1 on DCs (Fig. 3.3) [78]. In DC-T cell co-culture, OVA$_{323-339}$-specific Vβ5^{+} T cells cultured with PlxnA1$^{-/-}$ DCs did not proliferate compared to WT control DCs (Fig. 3.3B) [71, 78]. The effect of PlxnA1 deficiency on T cell proliferation was even observable at Day 2, an early time point, with this effect becoming much more pronounced as the cell proliferations were continually progressing through Day 4, and 7 (Fig. 3.3B). This pattern of inefficient Vβ5^{+} T cell proliferation in the absence of PlxnA1 was reiterated in the total T cell population (Fig. 3.3C). It should be noted that in both the total TCR^{+} and the Vβ5^{+} OVA-specific populations, a majority of T cells were proliferative even upon stimulation with PlxnA1$^{-/-}$ DCs, compared to those with nonpulsed WT DCs (Fig. 3.3B-C). However, a dramatic difference between groups with and without PlxnA1 became apparent when considering the number of cell divisions they had undergone. T cells cultured with PlxnA1$^{-/-}$ DCs were two or three cell cycles behind T cells stimulated with WT control DCs at Day 4 and 7. The lack of engagement between PlxnA1 and Sema6D accounts for inefficient T cell proliferation at the later time points, with DCs lacking PlxnA1 [79]. Furthermore, much greater numbers of the Vβ5^{+} T cells incubated with PlxnA1$^{-/-}$ DCs remained non-proliferative at Day 4 (WT 9.2%; KO 23.3%; No OVA 56.2%) and Day 7 (WT 0.4%; KO 6.3%; No OVA 61.4%). The percent of CD69^{+} T cells detected in the culture that contained PlxnA1$^{-/-}$ DCs was comparable to those with WT DCs, as opposed to T cells with no OVA control DCs, exhibiting few CD69^{+} T cells (45.7%; 40.0%; 0.6%) at Day 2 (Fig. 3.3C). This suggests that T cells interacting with PlxnA1$^{-/-}$ DCs were able to upregulate the early activation marker, CD69, through TCR stimulation and co-stimulations, but were not

fully activated to undergo efficient proliferation due to the lack of a stimulation through PlxnA1.

Since the $CD3^+$ T cells used in co-culture experiments contained both $CD4^+$ and $CD8^+$ T cells, $CD8^+$ T cells were subsequently analyzed separately. $CD8^+$ T cells included in the T cell preparation showed no proliferation but a great reduction in total numbers, suggesting that $CD8^+$ T cells do not proliferate in response to OVA-pulsed BMDCs, and die off due to lack of TCR stimulation (data not shown). This result confirms that the target cells of OVA-pulsed BMDCs are OVA-specific $CD4^+$ T cells, as originally established [251].

The current co-culture experiments using $PlxnA1^{-/-}$ DCs not only verified the results of our PlxnA1-Fc blocking assays, but also revealed the impairment in T cell proliferation resulting from the complete lack of PlxnA1 on DCs, as opposed to the partial blocking of PlxnA1 interaction with competitive inhibition (Fig. 3.2 and 3.3).

$PlxnA1^{-/-}$ DCs are less effective in stimulating T cells in vivo.

We have confirmed that PlxnA1 on DCs augments T cell priming as shown by *in vitro* DC-T cell co-culture (Fig. 3.2C and 3.3B-C). To test whether the lack of PlxnA1 on DCs would cause a defect in T cell response under more physiologically relevant conditions, *in vivo* T cell activation experiments were performed by employing adoptive transfer. $PlxnA1^{-/-}$ or WT DCs that were OVA-pulsed were intravenously transferred into the tail vein of Ly5.1 congenic mice along with CFSE-labeled OT-II T cells at a 1:4 ratio. DCs left unpulsed were also transferred with OT-II T cells, as negative controls.

Analysis of the $V\beta5^+$ T cells gated out of the viable $Ly5.2^+$ TCR^+ populations at 72 hr. post-transfer revealed that a significantly reduced number of cells had undergone cell

divisions among the $V\beta5^+$ T cells injected with $PlxnA1^{-/-}$ DCs compared to those with the WT DC counterparts (WT 52.1%; KO 30.8%; No OVA 6.5%) (Fig. 3.4A). Dividing cells in both groups underwent similar division cycles, but the number of cells in each cell cycle with $PlxnA1^{-/-}$ DCs was consistently reduced compared to that with WT (WT 1.9%, 3.1%, 6.1%, 7.2%, 9.3%, and 24.5% vs KO 1.0%, 1.8%, 2.0%, 3.7%, 6.5%, and 15.8% in the 2^{nd} -7^{th} peak, respectively) (Fig. 3.4A, left and middle panels). T cells in both groups were still proliferating compared to those with no OVA-pulsed control DCs (Fig. 3.4A). *In vivo* T cell activation assay suggests that significantly fewer T cells were activated in the presence of DCs lacking PlxnA1.

A defect of $PlxnA1^{-/-}$ DCs in priming T cells was also observed by significantly reduced numbers of IFN-γ producing cells upon OVA restimulation, *ex vivo*, using an IFN-γ ELISPOT assay. Each spot represents a single IFN-γ producing cell, and 186 spots per 1.25 x 10^5 splenocytes on average were obtained from the mice exposed to WT DCs versus 57 spots to $PlxnA1^{-/-}$ DCs (Fig. 3.4B). We also observed a more than five-fold decrease (0.5% vs 3.2%) in IFN-γ producing cells in the $Ly5.2^+$ $V\beta5^+$ viable populations upon OVA-rechallenge when primarily stimulated by $PlxnA1^{-/-}$ DCs compared to WT controls, using intracellular IFN-γ staining and flow cytometry (data not shown). Significantly fewer IFN-γ-producing cells were generated upon stimulation by DCs lacking PlxnA1, suggesting that PlxnA1 is likely to play a role in inducing a Th1 response. However, despite the reduced numbers of IFN-γ producing cells that were attributable to the lack of PlxnA1 on DCs, the remaining cells that do not produce IFN-γ in our assay might have produced other cytokines. Therefore, cytokine analysis should be considered for a broad range of cytokines including the ones that induce either Th2 (IL-13 or IL-4), or Th17 (IL-23) response.

PlxnA1$^{-/-}$ DCs exhibit reduced migration in vitro.

Previously, RNAi was utilized to deplete PlxnA1 in DCs, through the introduction of retroviral short hairpin RNA [71, 78]. Although RNAi was successful in depleting PlxnA1, the retroviral transduction induced DC activation, implicating changes in their migration characteristics. This effect limited the utility of migration assays in analyzing the role of PlxnA1 in DC function. In addition, difficulties were encountered in obtaining a sufficient number of virus-infected DCs, in the process of depleting PlxnA1 by RNAi. These problems were completely circumvented by utilizing the *plxna1*$^{-/-}$ mice obtained from our collaborators [243].

Utilizing this model, we were able to observe significant reductions in PlxnA1$^{-/-}$ DC migration towards CCL19 (ELC) and CCL21 (SLC) compared to WT controls, in a trans-well migration assay (Fig. 3.5A-B). Migrating cells were quantified by a XTT assay by virtue of the characteristic of XTT-tetrazolium, which is reduced to orange-colored formazan by cellular metabolic activity. DCs with or without PlxnA1 were also tested with CXCL12 (SDF-1α), a chemokine, suggested to support DC migration to DLNs [242]. Although we consistently observed lower numbers of PlxnA1$^{-/-}$ DCs that had migrated towards CXCL12 compared to their WT counterparts, variability in cells migrating towards CXCL12 was much greater than towards the cytokines CCL19 or 21 (Fig. 3.5C). Since DCs used for the assay were TNF-α-treated mature DCs, neither PlxnA1$^{-/-}$ DCs nor WT DCs migrated towards CCL5 due to downregulation of its receptors which include CCR1 and 5, upon DC maturation (Fig. 3.5D) [200, 210]. We also examined the migration of PlxnA1$^{-/-}$ DCs towards C5a, a complement factor, to serve as a control. C5aR is also downregulated upon DC maturation, resulting in very low numbers of DCs migrating towards C5a (Fig. 3.5E)

[200]. In order to verify whether comparable numbers of PlxnA1$^{-/-}$ DCs and WT DCs were loaded for migration assays, both populations were subjected to XTT assays using serial dilutions starting from 1 x 10^5 cells. The control XTT assay samples provided means to deduce the number of cells from the optical density values determined by spectrophotometry. Based on this conversion, cell numbers were found to be equivalent between PlxnA1$^{-/-}$ DCs and their WT counterparts (Data not shown). Based on the XTT assay, approximately up to 25% of the WT DCs migrated to the bottom well at the optimal concentration of CCL19 (1 μg/ml), CCL21 (2 μg/ml) and CXCL12 (2 μg/ml), compared to less than 5% of the PlxnA1$^{-/-}$ DCs (Fig. 3.5A-C).

To determine whether the chemokine receptor expressions are affected by the lack of PlxnA1 on DCs, the expression levels of CCR7 and CXCR4, receptors for CCL19/21 and CXCL12, respectively, were examined. The transcript levels and surface expression of CCR7 and CXCR4 were compared between PlxnA1$^{-/-}$ and WT DCs by real-time quantitative PCR (Fig. 3.5F) and by flow cytometry using surface staining with anti-CCR7-PE or anti-CXCR4-PE along with anti-CD11c-APC (Fig. 3.5G), respectively. Data from the quantitative PCR and the flow cytometry on Day 12-harvested BMDCs demonstrate that PlxnA1$^{-/-}$ and control DCs have the comparable levels of chemokine receptors, CCR7 and CXCR4 (Fig. 3.5F-G). Transcript levels of CCR7 and CXCR4 in PlxnA1$^{-/-}$ DCs were found to exhibit considerable variability compared to WT controls, but ultimately the differences in expression of both chemokines in PlxnA1$^{-/-}$ and WT control cells were proven insignificant ($p>>0.05$) (Fig. 2.5F). Therefore, PlxnA1 is not likely to be involved in the upregulation of these receptors but probably in the signaling upon chemokine stimulations for migration.

PlxnA1$^{-/-}$ DCs are less effective in migration to the draining lymph nodes.

We established that PlxnA1$^{-/-}$ DCs are unable to migrate towards defined chemokines *in vitro* (Figure 3.5). To determine whether the lack of PlxnA1 on DCs would result in defects in DC migration *in vivo*, equal numbers of differentially labeled PlxnA1$^{-/-}$ DCs and WT controls were subcutaneously co-transferred into the hind foot pads of C57BL/6Ly5.1 congenic mice. The major advantage of this strategy is that both populations are subjected to identical microenvironments.

Among the cells isolated from the popliteal DLNs, the transferred CD11c^{+} Ly5.2^{+} populations contained less PlxnA1$^{-/-}$ DCs compared to WT DCs from both sets of differentially labeled cells (CMRA^{+} WT vs CMFDA^{+} KO; CMFDA^{+} WT vs CMRA^{+} KO). Few CMRA^{+} or CMFDA^{+} cells were detected from non-DLNs (axillary) (data not shown). However, differences in the number of migrating cells between CMRA^{+} WT vs CMFDA^{+} PlxnA1$^{-/-}$ DCs were lower than the reverse experiment (CMFDA^{+} WT vs CMRA^{+} PlxnA1$^{-/-}$ DCs). The differences between CMRA^{+} WT vs CMFDA^{+} PlxnA1$^{-/-}$ DCs were found to be statistically insignificant ($p>0.05$), compared to the differences between CMFDA^{+} WT vs CMRA^{+} PlxnA1$^{-/-}$ DCs which were found to be siginificant ($p= \sim 0.01$). Although the data consistently showed that the number of PlxnA1$^{-/-}$ DCs migrating to the DLNs was less than their WT counterparts, there were samples that exhibited fewer migrating DCs regardless of the PlxnA1 status of the cells (Fig. 3.6). The question of whether DCs lacking PlxnA1 have a defect in migration *in vivo* upon challenge or maturation signals will have to be addressed by alternative strategies.

Rap1 is downstream of the PlxnA1 pathway in DCs.

Several observations have facilitated our hypothesis of a role of PlxnA1 in the regulation of Rap1 upon T cell conjugation. First of all, Rap1 has been found to function downstream of R-Ras signaling in a macrophage-like cell line [123]. In addition, PlxnA1 with its possible GAP activity towards R-Ras based upon sequence homology with other R-RasGAPs, suggests that Rap1 might be downstream of PlxnA1. Secondly, a Rap1 effector molecule called Arap3, has been shown to perform a RhoGAP function to suppress Rho activity [246]. Therefore, Rap1 would generate a negative signal for Rho activation, which was observed in the presence of PlxnA1 on DCs upon T cell conjugation, as shown in Chapter II [78].

In order to determine whether Rap1 is regulated by PlxnA1 upon T cell engagement, the level of activated Rap1 was measured in PlxnA1$^{-/-}$ DCs and their WT counterparts upon conjugation with T cells, by employing a GTP-Rap1 pull-down assay. As shown in Figure 3.7, our preliminary result demonstrates that PlxnA1$^{-/-}$ DCs contain an elevated level of activated Rap1 compared to WT DCs. In fact, WT DCs exhibited levels lower than those observed in control DCs that were left unpulsed (-OVA) or DCs maintained in the absence of T cell conjugation (Fig. 3.7). Since the OT-II T cells were fixed in 1% formaldehyde prior to the conjugation, there was no contribution of activated Rap1 from T cells. The reduction in activated Rap1 levels upon WT DC-T cell conjugation suggests that Rap1 activation is suppressed by PlxnA1. In addition, baseline Rap1 activity in PlxnA1$^{-/-}$ DCs appears elevated upon T cell engagement, compared to the basal level of Rap1 activity under unstimulated conditions.

Based on the assumption that PlxnA1 can exhibit R-RasGAP activity, the reduction of downstream Rap1 activity upon T cell conjugation would be due to R-Ras function suppressed by the GAP activity of PlxnA1 [32, 105, 123]. The reduction of Rap1 activity upon T cell conjugation, in the presence of PlxnA1 on DCs, may explain the previous observation demonstrating that Rap1 suppresses Rho via one of its effectors [246].

Discussion

PlxnA1 is expressed on myeloid DCs.

Among the different subsets of freshly isolated DCs and bone marrow-derived DCs, along with other cell types including B and T lymphocytes, PlxnA1 was detected primarily in myeloid DCs, especially in *in vitro* cultured BMDCs, by real-time quantitative PCR (Fig. 3.1). The expression of PlxnA1 in the myeloid cell types was evident from the lack of a similarly pronounced signal in splenic B and T lymphocytes.

PlxnA1 transcription was not detected in freshly isolated or *in vitro* Flt3L-induced ($CD11c^{+}$ $mPDCA\text{-}1^{+}$ $B220^{+}$) pDCs isolated from bone marrow. This result is in stark contrasts to a previous report suggesting significant levels of PlxnA1 transcription in Flt3L-induced pDCs, where it was functionally implicated in TLR7 and TLR9 signaling pathways [30]. Our unpublished data using freshly isolated pDCs from mouse bone marrow stimulated with known agonists for TLR7 and 9 also suggests a reduction in the Type I- IFN response in $PlxnA1^{-/-}$ pDCs compared to their WT counterparts (data not shown). Therefore, a means for detecting PlxnA1 protein is necessary, but unfortunately a currently available commercial antibody lacks specificity. Since transcript levels do not correlate with the amount of functional protein, surface expression as well as total protein expression of PlxnA1 should also be determined using PlxnA1-specific monoclonal antibodies, which we are in the process of producing. Conversely, it is possible that low PlxnA1 transcript levels do not represent actual protein expression (Figure 3.1), because of the possibility of an extensive half-life of the protein. Irregardless, PlxnA1 appears to be expressed at much higher levels in myeloid lineages, especially in BMDCs, compared to other immune cells. Based on this

observation, we chose to study PlxnA1 function in mature BMDCs. The current study is based on our hypothesis that PlxnA1 must play an important role in cells that highly express the protein [175, 176, 178, 179]. We also expect that Th responses stimulated with BMDCs would be similar to those with myeloid DCs.

PlxnA1 plays a role in T cell priming upon conjugation.

Accumulated evidence suggests that PlxnA1 is involved in the events that occur during DC-T cell conjugation. In support of this role is the observation that Sema6D, a PlxnA1 ligand, is expressed on T cells [29, 79]. Experiments employing DC-T cell co-culture with PlxnA1-Fc blockage demonstrate that DCs lacking PlxnA1 intercellular activity exhibit only suboptimal T cell priming upon contact, resulting in inefficient T cell proliferation (Fig. 3.2C). The basic experiment indicates that the intercellular activity of PlxnA1 upon T cell engagement is essential for T cell priming. Even though there is the possibility of a direct stimulatory effect from PlxnA1-Fc, overall inhibition of T cell proliferation by PlxnA1-Fc suggests that activation of DCs through PlxnA1 needs to occur for optimal T cell priming.

We can conclude from this experiment that blocking PlxnA1 interaction with T cell components upon DC contact is sufficient to inhibit naïve T cell proliferation. That PlxnA1 affects T cell responses from the initial phase in supported by our previous observation that PlxnA1 is involved in F-actin polarization towards the interface upon T cell conjugation (Chapter II) [78]. Actin polarization in APCs has previously been shown to play a crucial role in optimal conjugation with T cells and subsequent T cell responses [91, 92, 233, 244].

In addition, our co-culture experiments with PlxnA1$^{-/-}$ DCs demonstrate that T cells are not fully activated to undergo efficient proliferation and clonal expansion due to the lack of a

stimulation through PlxnA1 (Fig. 3.3A-B). However, T cells stimulated by DCs lacking PlxnA1 are still able to upregulate the early activation marker, CD69, through TCR stimulation and co-stimulations (Fig. 3.3B).

T cell stimulation by DCs lacking PlxnA1 not only confirmed the results of the PlxnA1-Fc blocking assays, but also demonstrated the grave consequences to T cell proliferation resulting from the complete lack of PlxnA1 on DCs, as opposed to the partial blocking of PlxnA1 interaction with competitive inhibition (Fig. 3.2C and 3.3B-C). Since previous studies have shown no defect of PlxnA1$^{-/-}$ DCs in antigen uptake or processing as well as in antigen binding affinity of MHC Class II molecules on the cell surface, the impact of PlxnA1 on T cell priming is attributable to the role of PlxnA1 upon DC-T cell engagement. Furthermore, PlxnA1 has been shown to perform cis-interactions with a complex of Trem-2/DAP12 surface proteins which is known to transduce a mitogenic signal through the Erk pathway [29, 252]. Since DAP12 activation was also shown to occur in the context of Sema6D-PlxnA1 ligation, it supports our findings that the lack of PlxnA1 on DCs leads to inefficient proliferation of cognate T cells, although Sema6D does not appear solely responsible for the stimulatory activity of PlxnA1 especially in the early phases of T cell activation [29, 79].

OT-II T cell activation by DCs lacking PlxnA1 is reduced in vivo.

The question of whether the lack of PlxnA1 on DCs would cause a defect in T cell responses under more physiologically relevant conditions was addressed by our *in vivo* T cell activation experiments employing adoptive transfer. *In vitro* co-cultures suggested that proliferation of T cells stimulated with DCs lacking PlxnA1 was not as efficient as those with

WT DCs (Fig. 3.3). *In vivo* T cell activation assays suggest that a significantly reduced number of T cells were activated in the presence of DCs lacking PlxnA1. The exogenous $Ly5.2^{+}$ TCR^{+} T cells co-transferred with $PlxnA1^{-/-}$ DCs in the spleen of each recipient were also significantly fewer than those with WT DCs, as determined by exogenous $V\beta5^{+}$ T cell staining, suggesting that more T cells might have died due to the lack of optimal stimulation by co-transferred $PlxnA1^{-/-}$ DCs. The question of whether PlxnA1 plays a role in T cell survival is an interesting one and should be addressed in future studies. Nontheless, DCs lacking PlxnA1 did not produce as a vigorous T cell proliferation as WT controls *in vivo* (Fig. 3.4A). In addition, the lack of OVA-specific $V\beta5^{+}$ T cell proliferation in the presence of unpulsed control DCs supports the accepted model of antigen-dependent *in vivo* T cell activation by OVA-stimulated BMDCs (Fig. 3.4A, the right panel).

A defect of $PlxnA1^{-/-}$ DCs in priming T cells was also uncovered by significantly fewer number of IFN-γ-producing cells upon OVA restimulation, *ex vivo,* using an IFN-γ ELISPOT assay (Fig. 3.4B). Since the ELISPOT assay cannot specifically measure exogenous OVA-specific T cells, flow cytometric analysis using intracellular IFN-γ staining was also performed. There was a more than five-fold reduction in the number of IFN-γ-producing cells in the exogenous OVA-specific T cell population upon OVA-re-challenge when formerly stimulated by $PlxnA1^{-/-}$ DCs compared to WT controls (data not shown). Significantly fewer IFN-γ-producing cells were generated upon stimulation when they were originally activated by DCs lacking PlxnA1, suggesting that PlxnA1 plays a role in inducing a Th1 response. Indirect evidence suggested that PlxnA1 might favor a Th1 response through the production of IL-12p70, based on the findings that only when PlxnA1 was present on DCs, a higher level of IL-12 was detected from DCs upon stimulation by Sema6D,

a PlxnA1 ligand, [29, 180]. However, the cells that did not produce IFN-γ in our assay might have produced other cytokines, inducing a Th2 (IL-13 or IL-4), or a Th17 (IL-23) response. Thus, cytokine profiling upon DC-T cell co-culture utilizing cytokine arrays followed by ELISA and/or ELISPOT assays will address the question of whether PlxnA1 on DCs plays a role in the fate-determination of cognate T cells. The results should then be verified by additional ELISPOT assays for various cytokines on *ex vivo* re-challenged splenocytes isolated from the recipients after adoptive transfer. An additional question to be answered is what Th response occurs in the absence of PlxnA1 [253].

PlxnA1$^{-/-}$ DCs exhibit reduced migration.

PlxnA1 is a well-known path-finder in neurons for axonal growth in response to a soluble guidance cue, Sema3A [4, 34, 140, 243]. This is the primary reason why PlxnA1 has been suspected to play a role in chemokine-induced DC migration. However, no impairment was previously observed in DC migration to the DLNs in *plxna1*$^{-/-}$ animals [29]. Although skin painting with FITC is a commonly used method, it harbors some disadvantages in measuring DC migration *in vivo*, for the following reasons: 1) Free FITC can be carried over from the skin and taken up by resident DCs in DLNs [208, 212]; 2) The skin tissue treated with FITC contains complex subsets of DCs including Langerhans cells and dermal DCs which are still poorly characterized [208, 212], and 3) Unidentified immune defects in *plxna1*$^{-/-}$ animals could interfere with DC behavior. To address these shortcomings, we employed two different strategies: 1) We examined the *in vitro* DC migratory capacity towards defined chemokines; and 2) We analyzed *in vivo* migration of PlxnA1$^{-/-}$ DCs and

WT controls into the DLNs by the application of differential labeling and subsequent subcutaneous co-transfer into congenic recipient mice.

PlxnA1$^{-/-}$ DCs exhibited a more than 50% reduction in migration towards chemokines, CCL19 (ELC) and CCL21 (SLC), compared to WT controls (Fig. 3.5A-B). Despite larger variations in migratory activity towards CXCL12, we also observed reduced migration of PlxnA1$^{-/-}$ DCs towards CXCL12, compared to WT DCs (Fig. 3.5C). Irregardless of PlxnA1 status, few DCs migrated towards CCL5. This result was expected since DC maturation results in the downregulation of its receptors, CCR1 and 5 (Fig. 3.5D). Analysis of transcript levels and surface expression of CCR7 and CXCR4 in PlxnA1$^{-/-}$ and WT DCs revealed that expression of these chemokine receptors is not affected by PlxnA1 status of the cells (Fig. 3.5F-G). Therefore, PlxnA1 is most likely not involved in the upregulation of these receptors but probably in the signaling induced upon chemokine stimulations for migration.

The question of whether the lack of PlxnA1 on DCs would affect migration *in vivo* was addressed by differential labeling of DCs from *plxna1*$^{-/-}$ and WT mice and by subsequent co-transfer into congenic Ly5.2^{+} mice. Despite the advantages of this strategy in that both populations are subjected to identical microenvironments, we encountered an unexpected problem associated with using the two different dyes (CMFDA and CMRA). Co-transfer following labeling of PlxnA1$^{-/-}$ DCs with CMRA and of WT DCs with CMFDA into the foot pads resulted in significantly reduced migration of PlxnA1$^{-/-}$ DCs to the DLNs, suggesting inefficient trafficking of PlxnA1$^{-/-}$ DCs. However, reversal of the staining (CMRA^{+} WT and CMFDA^{+} PlxnA1$^{-/-}$ DCs) resulted in reduced trafficking of the CMRA^{+} WT DCs compared to the CMFDA^{+} WT DCs to the DLNs. This resulted in the differences between CMRA^{+} WT versus CMFDA^{+} PlxnA1$^{-/-}$ DCs in migration being statistically insignificant ($p>0.05$),

whereas migration CMFDA^{+} WT versus CMRA^{+} PlxnA1$^{-/-}$ DCs was statistically different (p= ~ 0.01). A one possible explanation could be that CMRA is more toxic to cells than CMFDA, causing more cell death for CMRA^{+} DCs, *in vivo*. Indeed, when CMRA^{+} WT DCs and CMFDA^{+} PlxnA1$^{-/-}$ DCs migrated, the differences observed were less, possibly due to more cell death of the CMRA^{+} WT DCs (Fig. 3.6). When using CMRA^{+} PlxnA1$^{-/-}$ DCs and CMFDA^{+} WT DCs, the differences observed were greater, also possibly due to higher cell death of CMRA^{+} PlxnA1$^{-/-}$ DCs. Nontheless, the data consistently showed that the numbers of PlxnA1$^{-/-}$ DCs migrating to the DLNs were less than their WT counterparts, although there were some samples in either group that had few migrated DCs regardless of their origins (Fig. 3.6).

To definitively answer the question of whether PlxnA1 deficiency results in defective DC trafficking *in vivo* upon challenge or maturation signals, alternative strategies should be employed. A different approach is to use a single fluorescent dye without any reported toxicity and label a single population, PlxnA1$^{-/-}$ or WT DCs, but leaving the other unlabeled. By virtue of the congenic mice expressing the Ly5 antigen, differences in migration are expected to be easily determined between labeled and unlabeled exogenous DCs.

Rap1 is downstream of the PlxnA1 pathway in DCs.

Previously, it was observed that the level of activated Rho GTPase is upregulated by PlxnA1 signaling upon T cell contact, resulting in subsequent actin polarization towards the interface with T cells (Chapter II) [78]. Actin polarization in APCs, especially DCs, is known to be critical for T cell stimulation [91, 92, 233, 244]. Rho GTPases (Rho A, B or C) do not appear to bind to PlxnA1, directly [32, 33]. In contrast, it is strongly suggested that R-

Ras GTPase interacts with PlxnA1 through its conserved domains for R-RasGAP. In addition, there is circumstancial evidence suggesting that PlxnA1 exhibits R-RasGAP activity [32, 105]. Rap1 has appeared as another potential player downstream of PlxnA1 since Rap1 can be activated by R-Ras [123]. Rap1 is also linked to Rho via Arap3 which suppresses Rho activation [246]. In line with R-Ras and Rho, the level of activated Rap1 in PlxnA1$^{-/-}$ DCs was verified to determine whether Rap1 is regulated by PlxnA1. By means of GST pull-down assays, the level of active Rap1 in PlxnA1$^{-/-}$ DCs was detected was greater than that of WT DCs, upon T cell engagement. Furthermore, the level of activated Rap1 in WT DCs upon T cell engagement was even reduced compared to its basal levels under resting conditions such as no stimulation by T cells or a specific antigen (Fig. 3.7). This finding suggests that Rap1 activity is suppressed by PlxnA1, as a consequence of the R-RasGAP activity of PlxnA1. It is also plausible that PlxnA1 might perform a GAP activity towards Rap1, based on the conserved SynGAP region of PlxnA1 which is known to be essential for RapGAP activity [32, 136]. Indeed, there is a group of the R-RasGAPs that also serve as RapGAPs [137].

Similar to R-Ras, Rap1 also signals for cellular adhesion and/or migration mainly by activating integrins [119, 121, 132, 245, 254]. Indeed, Rap1 is known to play a critical role in regulating cell migration and/or adhesion, in immune cells among others [125, 129-131, 133, 245, 250, 254, 255]. With regard to DCs, defective Rap1 signaling was found to cause impaired DC trafficking *in vivo* [125]. The physiological significance of suppressing Rap1 activity upon stimulation has been suggested for T cells [129, 131]. CD28 suppresses Rap1 activity while CD3 stimulates Rap1 activation, upon T cell activation by APCs. Rap1 downregulation by CD28 was shown to prevent activation-induced cell death or cell cycle

arrest which is mediated through a hyperactive LFA-1-ICAM-1 interaction. Hyperactive Rap1 activity resulted in reduced Erk activation, concomitant with upregulation of $p27^{kip1}$, an indicator of cell cycle arrest, leading to less IL-2 production by T cells [129]. Therefore, a potential suppressive role of PlxnA1 on Rap1 activity could be a part of PlxnA1's function in promoting an optimal T cell response, by regulating integrin activation.

Regardless of R-Ras involvement, it should be verified whether the Rap1 activity is suppressed by PlxnA1 upon T cell conjugation, and whether this results in an elevated level of Rho activation. Suppression of Rap1 activity by PlxnA1 upon T cell conjugation is anticipated not to only result in elevated levels of Rho activity leading to actin remodeling and polarization but also in the regulation of integrin activities through effector molecules such as RapL which are responsible for cell adhesion and/or migration. Ultimately, the study of Rap1 activity regulation by PlxnA1 should be extended to cover the events involved in chemokine-induced DC migration.

Figure 3.1. PlxnA1 transcript levels determined by real-time PCR: PlxnA1 RNA transcript levels were measured as follows. 1) BMDCs or Mac: Twelve day-cultured mature BMDCs derived from WT and *plxna1*$^{-/-}$ mice; BM-derived CD11b^{+} CD11c^{-} Mac; Flt3L-induced mPDCA-1^{+} CD11c^{+} B220^{+} pDCs ; and 2) freshly isolated cells: CD11c^{+} CD11b^{+} mDCs from BM; mPDCA-1^{+} CD11c^{+} B220^{+} pDCs from BM; peritoneal cells; splenic CD11c^{+} CD8α^{-} mDCs; DC11c^{+} CD8α^{+} lDCs; and splenic CD3^{+} T cells and CD19^{+} B cells. Except for the PlxnA1$^{-/-}$ BMDCs as a negative control, all the other samples were prepared from WT C57BL/6J mice. Except Day12 BMDCs and peritoneal cells, each group of cells was sorted by FACS (MoFlo) for RT-PCR analysis. And PlxnA1 transcript levels were calculated and normalized β-actin levels. Each value represents fold increase over PlxnA1$^{-/-}$ DCs as 1. Data is representative of three independent experiments.

Figure 3.1

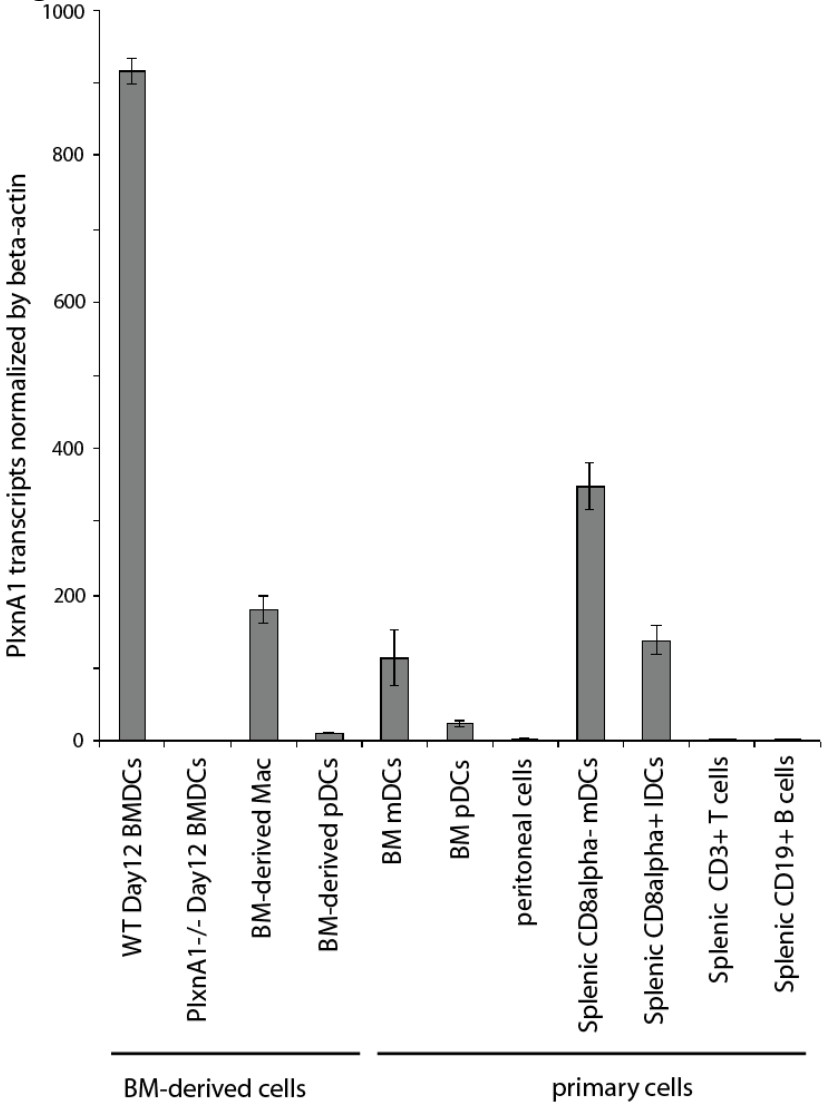

PlxnA1 transcripts normalized by beta-actin
1000
800
600
400
200
0
WT Day12 BMDCs
PlxnA1-/- Day12 BMDCs
BM-derived Mac
BM-derived pDCs
BM mDCs
BM pDCs
peritoneal cells
Splenic CD8alpha- mDCs
Splenic CD8alpha+ lDCs
Splenic CD3+ T cells
Splenic CD19+ B cells
BM-derived cells
primary cells

Figure 3.2. DC-T cell co-culture with PlxnA1-Fc blockade: The extracellular fragment of murine PlxnA1 cDNA fused to human IgG1 Fc (PlxnA1-Fc) and Fc only control with an IL-2 signal peptide sequences were expressed in HEK293 cells, and purified using protein A/G agarose beads (A). A Coomassie blue-stained SDS PAGE gel (Ba) and Western blot (Bb) performed with anti-human IgG-Fc-HRP show the purified PlxnA1-Fc protein (arrow: ~180 kDa) and Fc only control protein (arrow: ~25 kDa), respectively (B). For each DC-T cell co-culture experiment, PlxnA1-Fc or Fc only was added in the co-culture of BMDCs along with CFSE-labeled OT-II T cells at Day 0. Harvested cells were stained with Vβ5-APC and CFSE peaks were analyzed on channel FL1 among the cells gated out of $V\beta5^+$ live lymphocytes by flow cytometry (C).

Figure 3.2

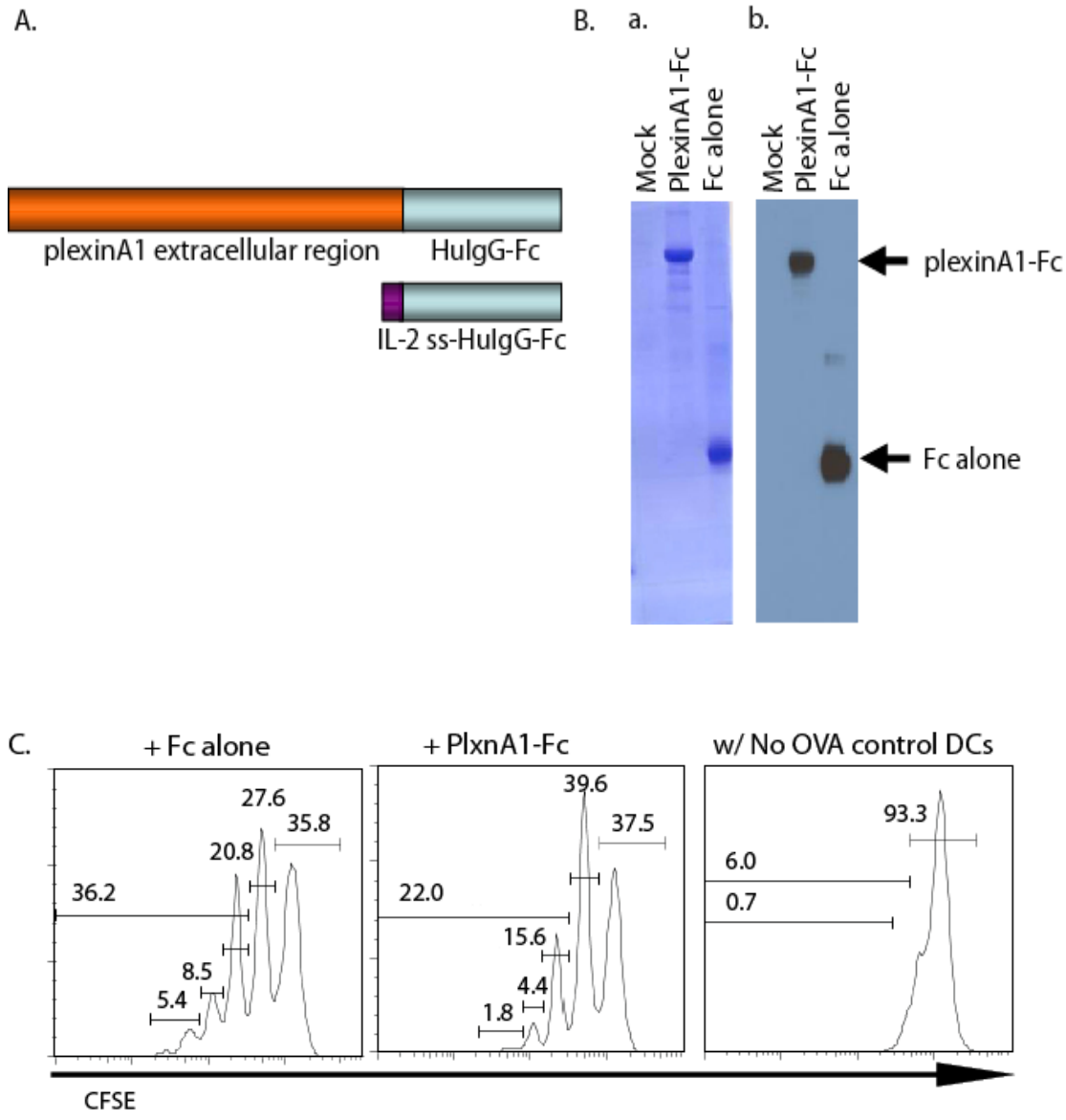

A.
plexinA1 extracellular region
HuIgG-Fc
IL-2 ss-HuIgG-Fc
B.
a.
b.
Mock
PlexinA1-Fc
Fc alone
Mock
PlexinA1-Fc
Fc a.lone
plexinA1-Fc
Fc alone
C.
+ Fc alone
+ PlxnA1-Fc
w/ No OVA control DCs
27.6
35.8
20.8
36.2
8.5
5.4
39.6
37.5
22.0
15.6
4.4
1.8
93.3
6.0
0.7
CFSE

Figure 3.3. DC-T cell co-culture using PlxnA1$^{-/-}$ DCs: BMDCs generated from *plxna1*$^{-/-}$ mice or WT littermates were pulsed with OVA protein at Day 12 for 12 hrs., and stained for mature DC surface markers, CD86, CD80, I-A^{b}, CD40 and CD11c (dark lines), using FITC-conjugated antibodies for I-A^{b}, PE-antibodies for CD86, CD80 and CD40, and APC-conjugated antibody for CD11c, to compare surface expression in PlxnA1$^{-/-}$ DCs with those on WT counterparts, respectively. Isotype controls were used with isotype-matched and fluorescent conjugate-matched hamster or rat antibodies (gray lines) to set a percentage of positive cells for desired marker expression in each sample gated out of total live populations (A). DCs from both groups were added in co-culture with CFSE-labeled OT-II T cells at 1:5 ratios, respectively. Cells were harvested at Day 2, 4, and 7, stained with anti-Vβ5-biotin or anti-CD69-biotin followed by SA-APC or anti-TCR-APC, and analyzed by flow cytometry. Plots were gated out of a live lymphocyte population, from which Vβ5-positive or Vβ5-negative populations were enumerated (B). Live lymphocyte populations from Day 2 samples was displayed with CFSE reduction and CD69 expression for Day 2 samples (C, the top row) and TCR expression for Day 4 and 7 (C, the middle and the bottom rows). One experiment representative of three independent experiments is shown.

Figure 3.3

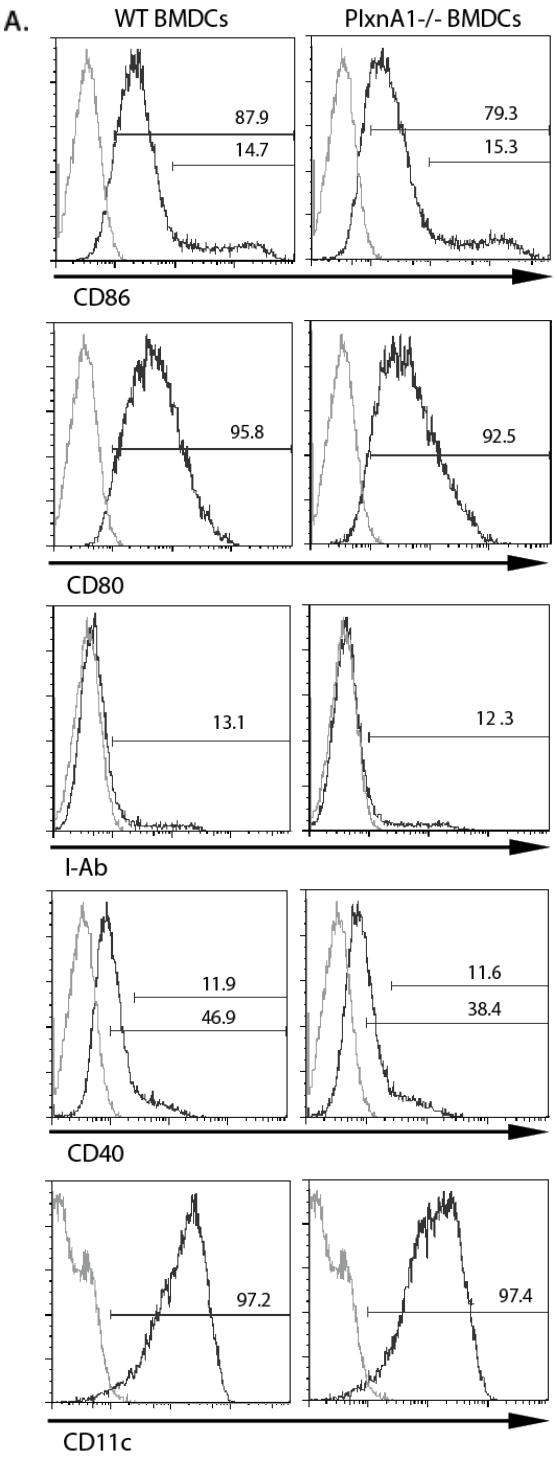

A.
WT BMDCs
PlxnA1-/- BMDCs
87.9
14.7
79.3
15.3
CD86
95.8
92.5
CD80
13.1
12 .3
I-Ab
11.9
46.9
11.6
38.4
CD40
97.2
97.4
CD11c

Figure 3.3

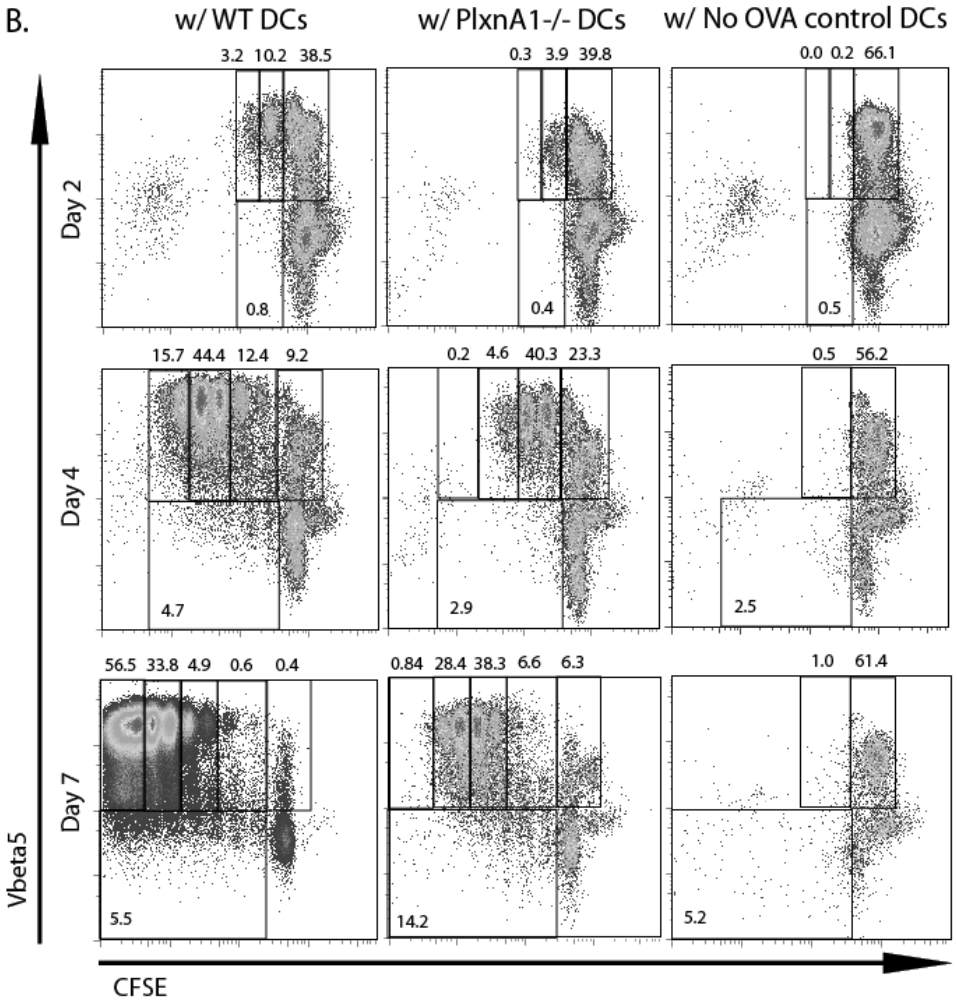

B.
w/ WT DCs
w/ PlxnA1-/- DCs
w/ No OVA control DCs
Day 2
Day 4
Day 7
Vbeta5
CFSE
3.2 10.2 38.5
0.8
0.3 3.9 39.8
0.4
0.0 0.2 66.1
0.5
15.7 44.4 12.4 9.2
4.7
0.2 4.6 40.3 23.3
2.9
0.5 56.2
2.5
56.5 33.8 4.9 0.6 0.4
5.5
0.84 28.4 38.3 6.6 6.3
14.2
1.0 61.4
5.2

Figure 3.3

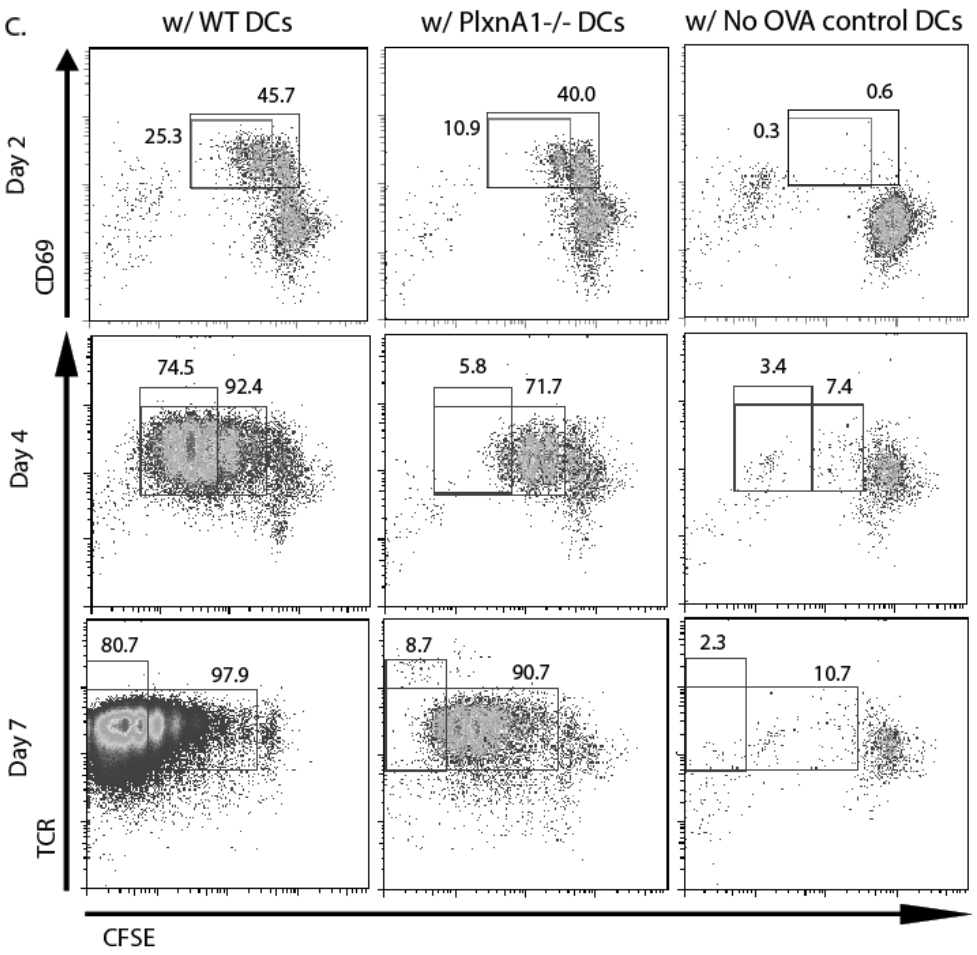
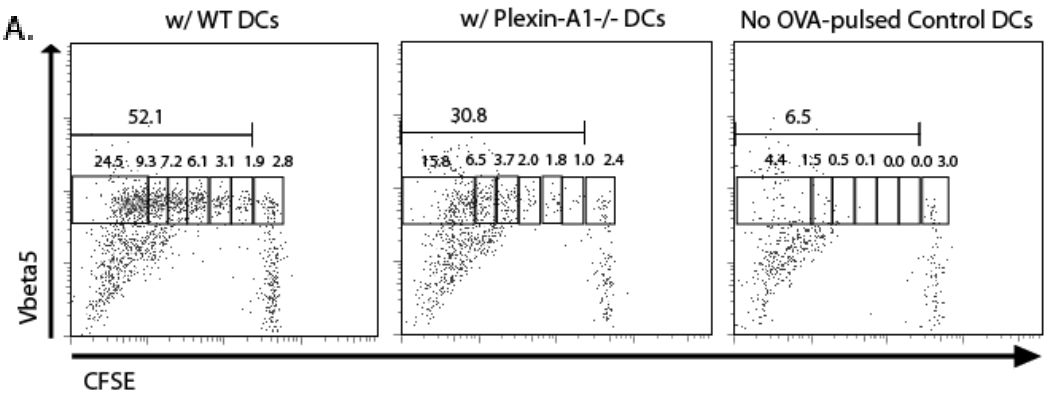

C.
w/ WT DCs
w/ PlxnA1-/- DCs
w/ No OVA control DCs
Day 2
CD69
45.7
25.3
40.0
10.9
0.6
0.3
Day 4
74.5
92.4
5.8
71.7
3.4
7.4
Day 7
TCR
80.7
97.9
8.7
90.7
2.3
10.7
CFSE

Figure 3.4. T cell activation assay *in vivo*: Day 12 BMDCs from *plxna1*$^{-/-}$ or WT littermates were pulsed with 0.1 μM peptide $OVA_{323-339}$, and intravenously transferred into the tail vein of B6-Ly5.1 congenic mice along with CFSE-labeled OT-II T cells at 1:4 ratios. The splenic T cells from each mouse were analyzed 72 hr. post-transfer. After staining with anti-Vβ5-biotin followed by SA-pacific blue along with anti-TCR-APC and anti-Ly5.2-APC-Cy7, CFSE-labeled $V\beta5^+$ T cells were gated out of $Ly5.2^+$ TCR^+ T cells and visualized by flow cytometry (A). IFN-γ production was determined by ELISPOT, 24 hr. post-restimulation with OVA, *ex vivo* (B). Each group reflects an experiment performed in triplicate and the figure is representative of two independent experiments. Statistical analysis was carried out by student t-test using software Prism.

Figure 3.4


A.
w/ WT DCs
w/ Plexin-A1-/- DCs
No OVA-pulsed Control DCs
52.1
24.5 9.3 7.2 6.1 3.1 1.9 2.8
30.8
15.8 6.5 3.7 2.0 1.8 1.0 2.4
6.5
4.4 1.5 0.5 0.1 0.0 0.0 3.0
Vbeta5
CFSE

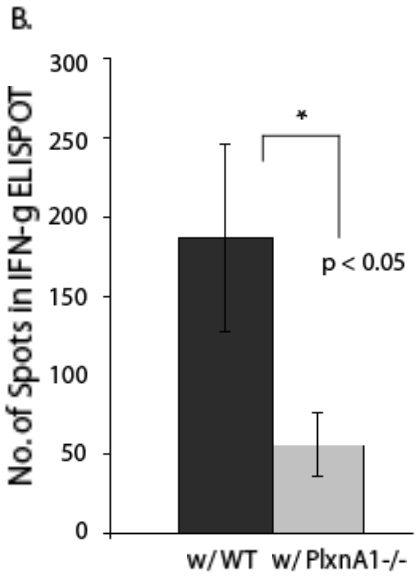

B.
No. of Spots in IFN-g ELISPOT
300
250
200
150
100
50
0
*
p < 0.05
w/ WT
w/ PlxnA1-/-

Figure 3.5. *In vitro* migration assay for PlxnA1$^{-/-}$ DCs: PlxnA1$^{-/-}$ DCs were tested for their migratory activities towards various chemokines, *in vitro* using 96-transwell plates. Day 12 BMDCs from *plxna1*$^{-/-}$ mice and from WT littermates were exposed to CCL19 (A), CCL21 (B), CXCL12 (C), CCL5 (D), and C5a (E, a complement factor as a negative control). Cells migrated towards each chemokine present in the bottom wells were measured at 450-650 nm using a XTT assay. For setting standard curves, PlxnA1$^{-/-}$ DCs along with WT DCs (1×10^5 cells each) were also subjected to XTT assays with serial dilutions at a 1:1 ratio. Standard curves served as internal controls for the equivalent numbers of WT and PlxnA1$^{-/-}$ DCs used for each assay. The average of triplicate wells reflecting one concentration of each chemokine is displayed with standard deviations. One representative of ten independent experiments is shown (A-E). The number of cells in each sample was deduced based on the equation aquired from either standard curve. Expression of CCR7 and CXCR4 was compared between PlxnA1$^{-/-}$ DCs and WT DCs, both from the preparations used for *in vitro* migration assays. Real-time PCR was performed on cDNA from PlxnA1$^{-/-}$ DCs or WT counterparts for Ccr7 and Cxcr4 along with PlxnA1 expression. Transcript levels were normalized to β-actin levels and expressed as fold over WT (F). Surface expression of CCR7 and CXCR4 along with that of CD11c^{+} was compared among the total live populations from PlxnA1$^{-/-}$ or WT BMDCs using anti-CCR7-PE, anti-CXCR4-PE, or anti-CD11c-APC antibodies (dark lines). Isotype controls were prepared with isotype-matched and fluorescent conjugate-matched hamster or rat antibodies (gray lines) to reflect the positive population that was gated out of total viable cell population (G). Data is representative of three independent experiments (F-G). Statistical analysis of the real-time PCR on CCR7 and CXCR4 between the two groups per set was carried out by student t test and Prism software.

Figure 3.5

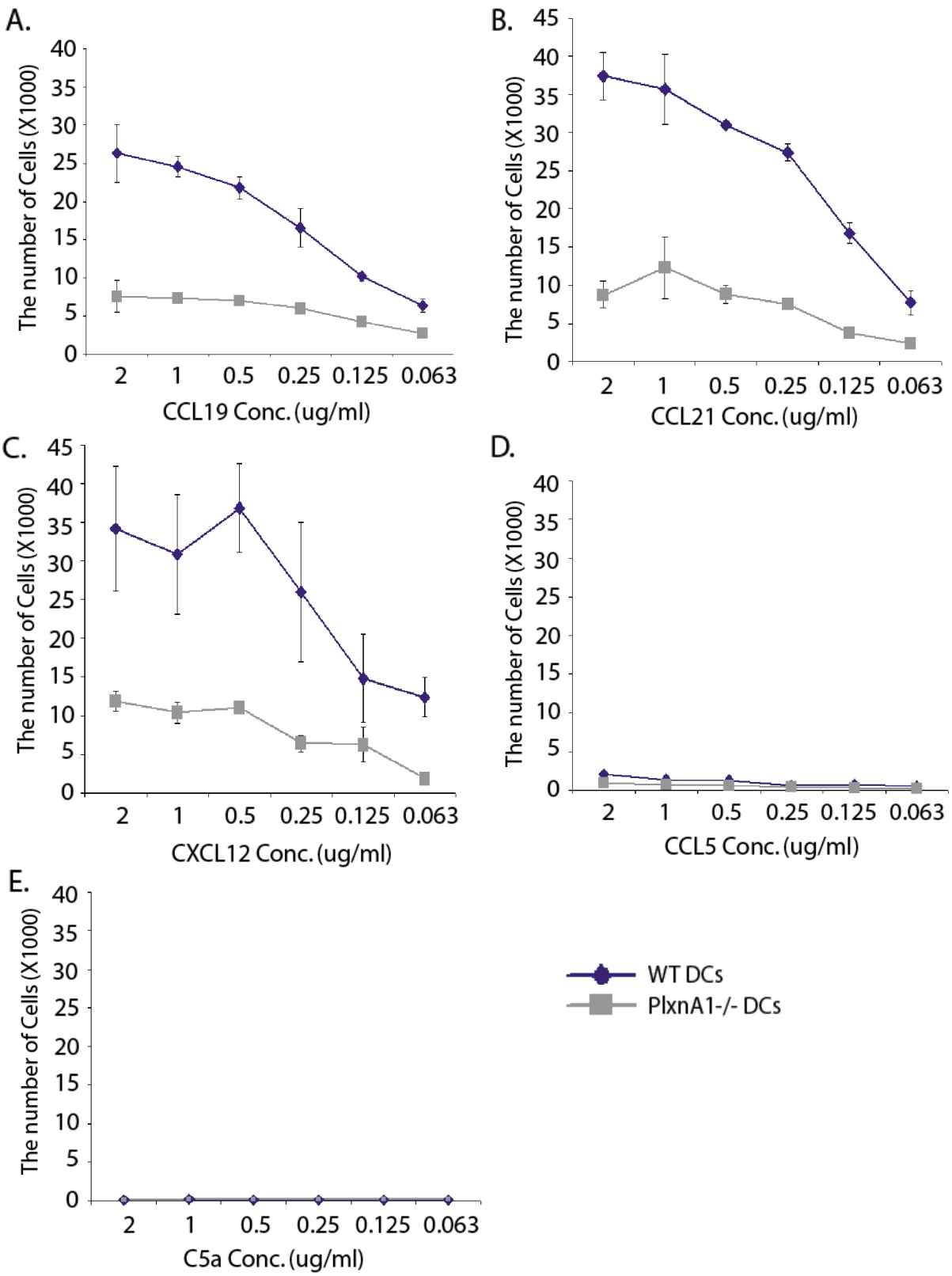

A.
The number of Cells (X1000)
40
35
30
25
20
15
10
5
0
2
1
0.5
0.25
0.125
0.063
CCL19 Conc. (ug/ml)
B.
45
40
35
30
25
20
15
10
5
0
The number of Cells (X1000)
2
1
0.5
0.25
0.125
0.063
CCL21 Conc. (ug/ml)
C.
45
40
35
30
25
20
15
10
5
0
The number of Cells (X1000)
2
1
0.5
0.25
0.125
0.063
CXCL12 Conc. (ug/ml)
D.
40
35
30
25
20
15
10
5
0
The number of Cells (X1000)
2
1
0.5
0.25
0.125
0.063
CCL5 Conc. (ug/ml)
E.
40
35
30
25
20
15
10
5
0
The number of Cells (X1000)
2
1
0.5
0.25
0.125
0.063
C5a Conc. (ug/ml)
WT DCs
PlxnA1-/- DCs

Figure 3.5

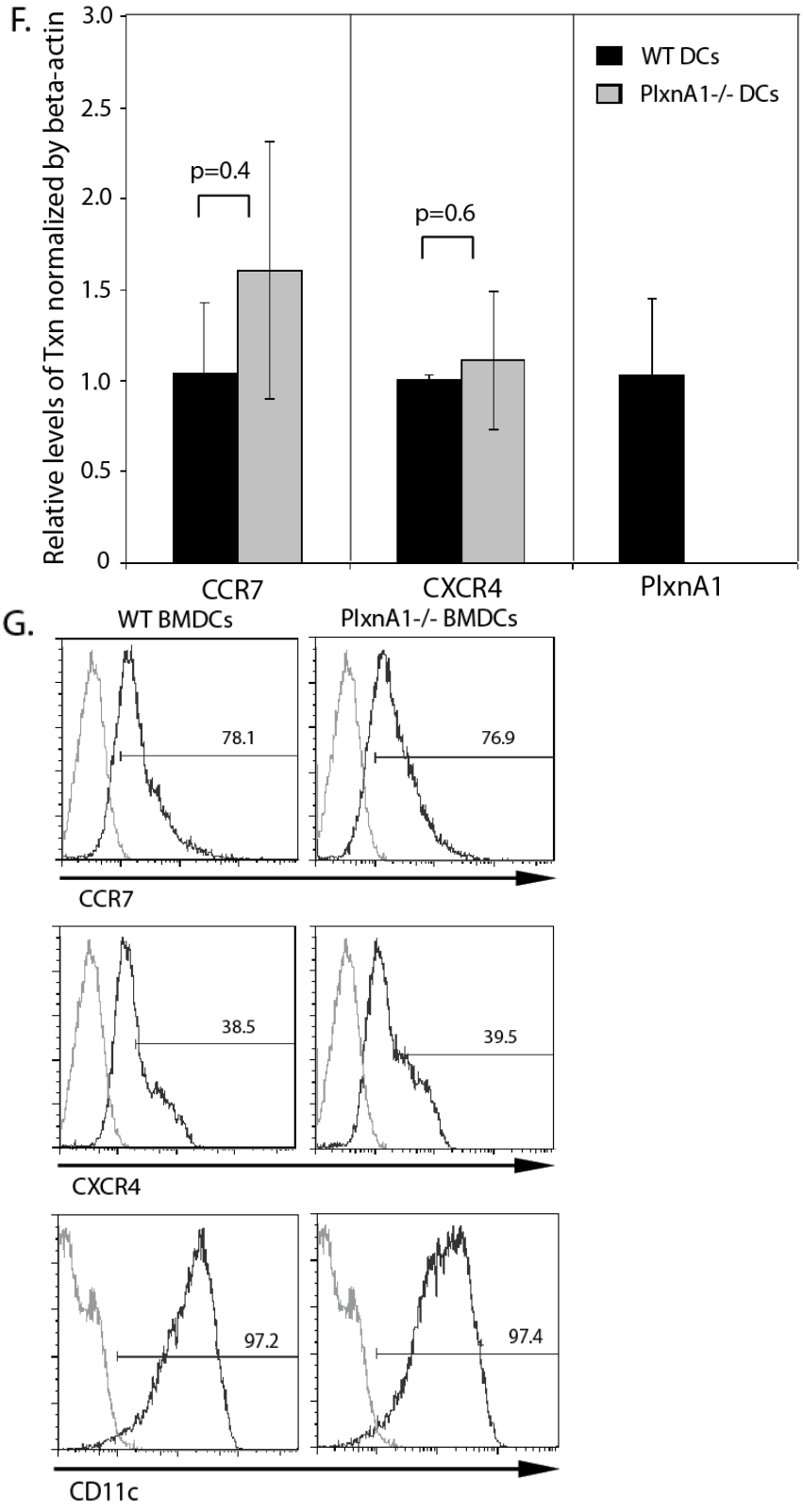

F.
Relative levels of Txn normalized by beta-actin
3.0
2.5
2.0
1.5
1.0
0.5
0
p=0.4
p=0.6
WT DCs
PlxnA1-/- DCs
CCR7
CXCR4
PlxnA1
G.
WT BMDCs
PlxnA1-/- BMDCs
78.1
76.9
CCR7
38.5
39.5
CXCR4
97.2
97.4
CD11c

Figure 3.6. *In vivo* migration assay for PlxnA1$^{-/-}$ DCs: PlxnA1$^{-/-}$ DCs were examined for their migration, from skin tissue to the DLNs. Day 12 BMDCs from *plxna1*$^{-/-}$ mice and WT littermates were OVA-pulsed, differentially labeled with CMRA and CMFDA, respectively, and mixed at a 1:1 ratio (1 x 10^6 cells each). As an internal control, the labeling was reversed. Equal numbers of differentially labeled PlxnA1$^{-/-}$ and WT DCs were subcutaneously transferred to the hind foot pads of Ly5.1 congenic mice. At 36 hr. post-transfer, DLNs (popliteal) and NDLNs (axillary) were isolated from the recipient mice and the total LN cells stained with anti-CD11c-APC-Cy7 and Ly5.2-APC. The transferred CMRA (FL2)$^+$ or CMFDA (FL1)$^+$ DC populations were analyzed in two-dimensional plots from cells gated out of a CD11c$^+$ Ly5.2$^+$ live population using flow cytometry. Either group is represented as percent CMFDA$^+$ or CMRA$^+$ cells from Ly5.2$^+$ CD11c$^+$ cells for each recipient's DLNs. Each open circle reflects a single recipient mouse. Data is representative of three independent experiments. Statistical analysis between the two groups per set was carried out by student t test and Prism software.

Figure 3.6

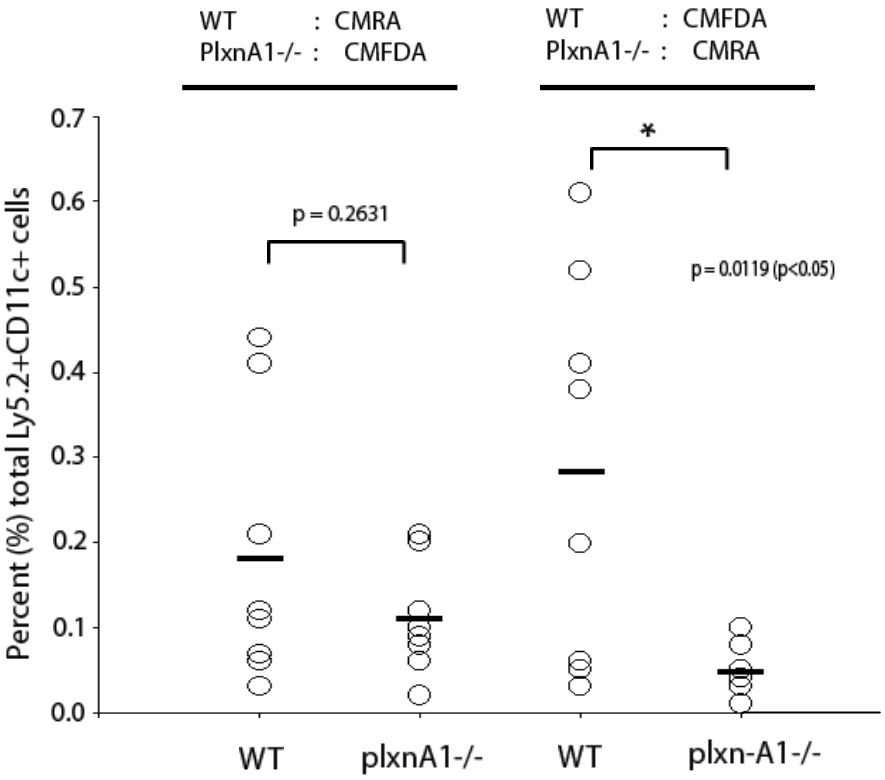
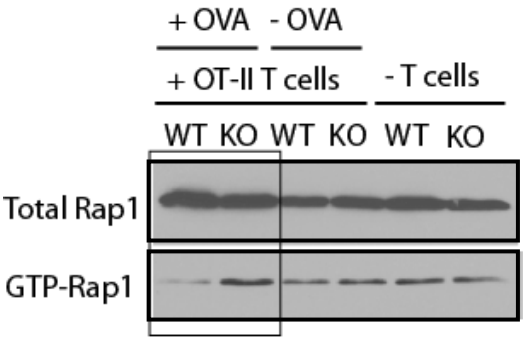

WT : CMRA
PlxnA1-/- : CMFDA
WT : CMFDA
PlxnA1-/- : CMRA
*
p = 0.2631
p = 0.0119 (p<0.05)
Percent (%) total Ly5.2+CD11c+ cells
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
plxnA1-/-
WT
plxn-A1-/-

Figure 3.7. GST pull-down assay for activated Rap1 in PlxnA1$^{-/-}$DCs upon DC-T cell conjugation. The amount of active Rap1 in PlxnA1$^{-/-}$ DCs upon T cell conjugation was compared to WT counterparts by GST pull-down assays. OVA-pulsed DCs (1 x 10^6) from *plxna1*$^{-/-}$ or WT mice were prepared. CD4$^+$ OT-II T cells (1 x 10^6) were isolated and fixed with 1% formaldehyde. Negative controls for antigen-dependent DC-T cell conjugation were non-OVA pulsed PlxnA1$^{-/-}$ or WT DCs with T cells as well as the ones pulsed with OVA but incubated without T cells. Subsequent to incubating each group of DCs with or without T cells at 37^{o}C for 2 min., GTP-bound Rap1 was precipitated with RBD-GST-agarose beads and detected along with total Rap1 from each cell lysate using western-blotting. GTP-Rap1 and total amounts of Rap1 from WT and PlxnA1$^{-/-}$ DCs are shown in the rectangular box.

Figure 3.7


+ OVA
- OVA
+ OT-II T cells
- T cells
WT KO WT KO WT KO
Total Rap1
GTP-Rap1

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

4.1. Finding PlxnA1 ligand on naïve T cells for T cell activation (Fig. 4.1; Fig. 5.1)

The work presented here shows that PlxnA1 on the surface of DCs is crucial for priming T cells upon DC-T cell conjugation, as well as for chemokine-induced DC migration. Although Sema6D on T cells has been identified as a PlxnA1 ligand (See Appendix), it is expected that PlxnA1 pairs with more than one ligand expressed on T cells in a spatiotemporal manner. It is because Sema6D is shown upregulated upon activation, while PlxnA1 is already expressed on mature DCs prior to encountering T cells, that I reasoned that there might be another ligand of PlxnA1 on naïve T cells [29, 71, 78, 79]. Identifying the PlxnA1 ligand expressed on naïve T cells that triggers intracellular signaling cascade upon cognate T cell engagement would allow us to examine more closely what molecular events take place upon activation of PlxnA1. When monoclonal antibody for PlxnA1 is available, we would like to perform high-throughput methods such as mass spectrometry, in order to identify Plxn-A1-interacting proteins from naïve T cells.

4.2. A role of PlxnA1 in T cell polarization (Fig. 4.2)

Our *in vitro* co-culture experiment using PlxnA1$^{-/-}$ DCs showed inefficient proliferation of cognate T cells stimulated by DCs lacking PlxnA1 not only in the earlier time point (Day 2) but also in the later stage (at Day 7), (See Fig. 3.3). Such data suggest that PlxnA1 might be involved in T cell polarization process. Extended function of PlxnA1 was also supported by a recent observation that Sema6D, PlxnA1 ligand, augments T cell response in the later stage of primary response [79]. Potential involvement of PlxnA1 in T cell polarization was suggested by a previous finding that PlxnA1 augments IL-12 secretion by DCs in response to Sema6D [29]. Moreover, the same report showed that IFN-γ production was significantly

reduced in *plxna1*$^{-/-}$ animals along with alleviated EAE symptoms upon MOG challenge [29]. Although recent evidence suggests that there are IL-12-independent pathways for induction of Th1 response, it is well studied that Th1 response is facilitated by IFN-γ which is strongly induced by IL-12 production from DCs in a variety of contexts [180, 183, 256-260]. In this regard, lower numbers of IFN-γ- producing cells in the OVA-recall response after *in vivo* T cell priming by PlxnA1$^{-/-}$ DCs, also implies that PlxnA1 favors inflammatory Th1 response (See Fig. 3.4). However, cytokine profiling should be valuable to determine whether PlxnA1 plays a role in terminal differentiation of T cells, since other cytokines inducing Th2 (IL-13 or IL-4), or Th17 (IL-23) response has not been tested. Cytokine arrays followed by ELISPOT/ELISA assays on DCs with or without PlxnA1 upon *in vitro* DC-T cell co-culture will provide a clue whether PlxnA1 on DCs affects T cell fates. Additionally, *in vivo* T cell priming followed by *ex vivo* antigen-recall response using ELISPOT assays will verify *in vitro* results. One additional question to that is what T cell response would be default in the absence of PlxnA1 [253].

4.3. PlxnA1 on migratory activity of DCs

Previously, no impairment was found in DC migration from the periphery to DLNs in *plxna1*$^{-/-}$ animals by others [29]. However, the methodology that they used entailing skin painting with FITC to measure DC migration *in vivo* has some disadvantages as mentioned elsewhere (Chapter III). Due to such drawbacks, it was imperative to re-examine the migratory properties of PlxnA1$^{-/-}$ DCs, and in the current study, we have performed *in vitro* and *in vivo* analyses. *In vitro* chemotaxis assays for defined chemokines including CCL21 and CCL19, PlxnA1$^{-/-}$ DCs showed significantly reduced capacity to migrate towards such

chemokines. However, there was no obvious difference in expression of CCR7 and CXCR4 in PlxnA1$^{-/-}$ DCs which are receptors for CCL21/19 and CXCL12, respectively, suggesting no role of PlxnA1 in regulating the expression of either receptor (See Fig. 3.5). Therefore, PlxnA1 is likely involved in the signaling upon chemokine-induced migration of DCs.

However, in the course of confirming whether inefficient DC migration upon chemokine stimulation would be physiologically relevant *in vivo*, we have encountered an unexpected complication with differential labeling. DC trafficking from the periphery to DLNs has been tested *in vivo* using differentially labeled DCs from *plxna1*$^{-/-}$ mice or WT littermates using two different fluorescent dyes, CMFDA and CMRA. Results showed that one pair of differentially labeled DCs (CMFDA^{+} PlxnA1$^{-/-}$ and CMRA^{+} WT)) found in recipient DLNs showed insignificant difference between PlxnA1$^{-/-}$ and WT DCs ($p>0.05$) compared to the other pair (CMRA^{+} PlxnA1$^{-/-}$ and CMFDA^{+} WT) of which much more CMFDA^{+} WT DCs were found in DLNs than CMRA^{+} PlxnA1$^{-/-}$ DCs ($p= \sim 0.01$) (See Fig. 3.6). In general, lower numbers of CMRA-labeled DCs were found in cells from DLNs than those of CMFDA-labeled ones.

One explanation would be that CMRA might have been more toxic than CMFDA, causing cell death. It is also possible that lack of PlxnA1 on DCs could have been compensated by other factors *in vivo*, such that a migratory defect of DCs did not appear as severe in animals as isolated DCs upon defined chemokines tested *in vitro*. In order to clarify whether the lack of PlxnA1 on DCs causes a severe defect in DC trafficking to DLNs upon challenge, an alternative strategy is to label PlxnA1$^{-/-}$ DCs or WT DCs with one fluorescent dye such as CMFDA which is thought to be minimally toxic. After transfer into

the periphery such as hind foot pads of congenic Ly5.1 recipient mice, both unlabeled and CMFDA-labeled Ly5.2^{+} DCs can be analyzed in comparison.

4.4. Small GTPases involved in PlxnA1 signaling (Fig. 4.3)

As previously shown, we have observed that PlxnA1 affects DC activity in chemokine-induced migration as well as in T cell priming upon conjugation. However, it is yet to be defined the molecular mechanism by which PlxnA1 signals for regulating such cellular behaviors. Previously, our group showed that DCs contained elevated levels of Rho GTPase activity only in the presence of PlxnA1 upon T cell engagement, followed by subsequent actin polarization towards the interface with T cells [78]. In the absence of PlxnA1, elevated Rho activity was not evident upon T cell conjugation, neither was actin polarization, which is known to be crucial for T cell response [91, 92, 233, 244]. However, there is still a gap between PlxnA1 and Rho GTPase, based on the evidence that Rho GTPases (RhoA, B, or C) do not directly bind to PlxnA1 [32, 33].

Instead, PlxnA1 has been suggested to interact with R-Ras, and perform R-RasGAP activity, upon Sema3A stimulation that requires downregulation of R-Ras activity [32, 105]. Although PlxnA1 has never been directly proven to exhibit such activity, R-RasGAP conserved sequence in the cytoplasmic region of PlxnA1 supports that this might be the case [32].

It is evident that R-Ras activates integrin signaling, for cellular migration and adhesion [119]. Related to R-Ras functionality, Rap1 has also been implicated in cell adhesion and migration, in the immune cells among others [124, 126, 130, 132, 250, 254, 255, 261-263]. Based on recent evidence, Rap1 functions downstream of R-Ras activity in macrophage-like

cell line [123]. In addition, Rap1 has a link to Rho via Arap3, a Rap1 effector protein, which performs RhoGAP activity, suppressing Rho activation [246]. Based on these links, we hypothesized that Rap1 might be regulated by PlxnA1.

To test our hypothesis, Rap1 activity has been compared in DCs in the presence or absence of PlxnA1 upon T cell conjugation using GST pull-down assays. Our preliminary attempts to compare Rap1 activity in PlxnA1$^{-/-}$ DCs to WT controls produced promising results, showing that a level of activated Rap1 in PlxnA1$^{-/-}$ DCs was elevated compared to WT DCs upon T cell contact (Fig. 3.7). This preliminary result suggests that PlxnA1 potentially down-regulates Rap1 activity. As suggested previously, it could be through its GAP activity for R-Ras, PlxnA1 suppresses R-Ras activation, which would results in downregulation of Rap1 activity. Another possibility is that PlxnA1 might play a GAP activity directly for Rap1 without involving R-Ras. This is less likely but still possible since PlxnA1 has SynGAP conserved region, known to be essential for RapGAP activity [136]. Ras and Rap proteins also share some of their GAPs [137, 264].

Regardless of whether R-Ras mediates PlxnA1-Rap1 pathway, our focus is to determine whether Rap1 downstream of PlxnA1 regulates activity of RhoA, as well, because RhoA was previously observed upregulated by PlxnA1 upon T cell interaction (Fig 2.4) [78]. As mentioned previously, a link between Rap1 and RhoA has been connected by a Rap1 effector molecule called Arap3, which is a RhoA-GAP, thereby suppressing RhoA activity [246]. In this way, downregulation of Rap1 activity by PlxnA1 which would result in elevated levels of RhoA activity (Fig. 2.4) [78]. Therefore, following the current experiments to verify suppression of Rap1 activity by PlxnA1, our aim is to determine whether Rho GTPase is suppressed by Rap1 upon T cell engagement. Downregulation of

Rap1 activity by PlxnA1 could be mimicked by introducing a known Rap1GAP protein into PlxnA1$^{-/-}$ DCs via transduction of adenoviral particles that are available through collaborations. Our hypothesis will be tested whether reduction of Rap1 activity executed by this exogenous Rap1GAP protein in the absence of PlxnA1 would lead to subsequent elevation of Rho activity. Furthermore, Rap1 functionally interacts with integrins, by reciprocally activating each other through its effector molecules such as RapL [130, 245, 254, 265].

Collectively, it appears that Rap1 would signal through Rho for actin remodeling, but also signal for integrin activation through its distinct effector molecules, in order to regulate cell adhesion and/or migration. Therefore, analogous to other contexts, Rap1 potentially regulated by PlxnA1 upon T cell conjugation is also likely to signal for activation of integrins as well as for actin remodeling in DCs, both of which are thought to contribute to optimal stimulation of T cell response. If this hypothetical pathway of PlxnA1-Rap1 is definitively proven, PlxnA1 relationship with Rap1 should be further tested in chemokine-induced DC migration, as well.

4.5. PlxnA1: its clinical implications

It is clearly shown that PlxnA1 on the surface of DCs is crucial not only in T cell priming but also in chemokine-induced DC migration. PlxnA1 is also implicated in augmentation of IFN-γ production, which is indicative of Th1 inflammatory response [180, 183, 256-260]. Such evidence that PlxnA1 is a stimulatory factor for T cell-mediated immunity proposes that PlxnA1 is a potential target for developing therapeutics against autoimmune inflammatory disorders such as multiple sclerosis (MS). Indeed, in EAE model

mimicking human MS, *plxna1*$^{-/-}$ mice showed significantly lower clinical morbidity scores along with profound reduction of IFN-γ production, compared to WT individuals, upon challenge [29].

Therefore, the mechanism of PlxnA1 signaling should be dissected including identification of its ligand as well as its effector molecules. Understanding PlxnA1 signaling mechanism will direct to a way of efficiently attenuating PlxnA1 signaling *in vivo* without causing significant side effects, for example, through the usage of PlxnA1-Fc blockade.

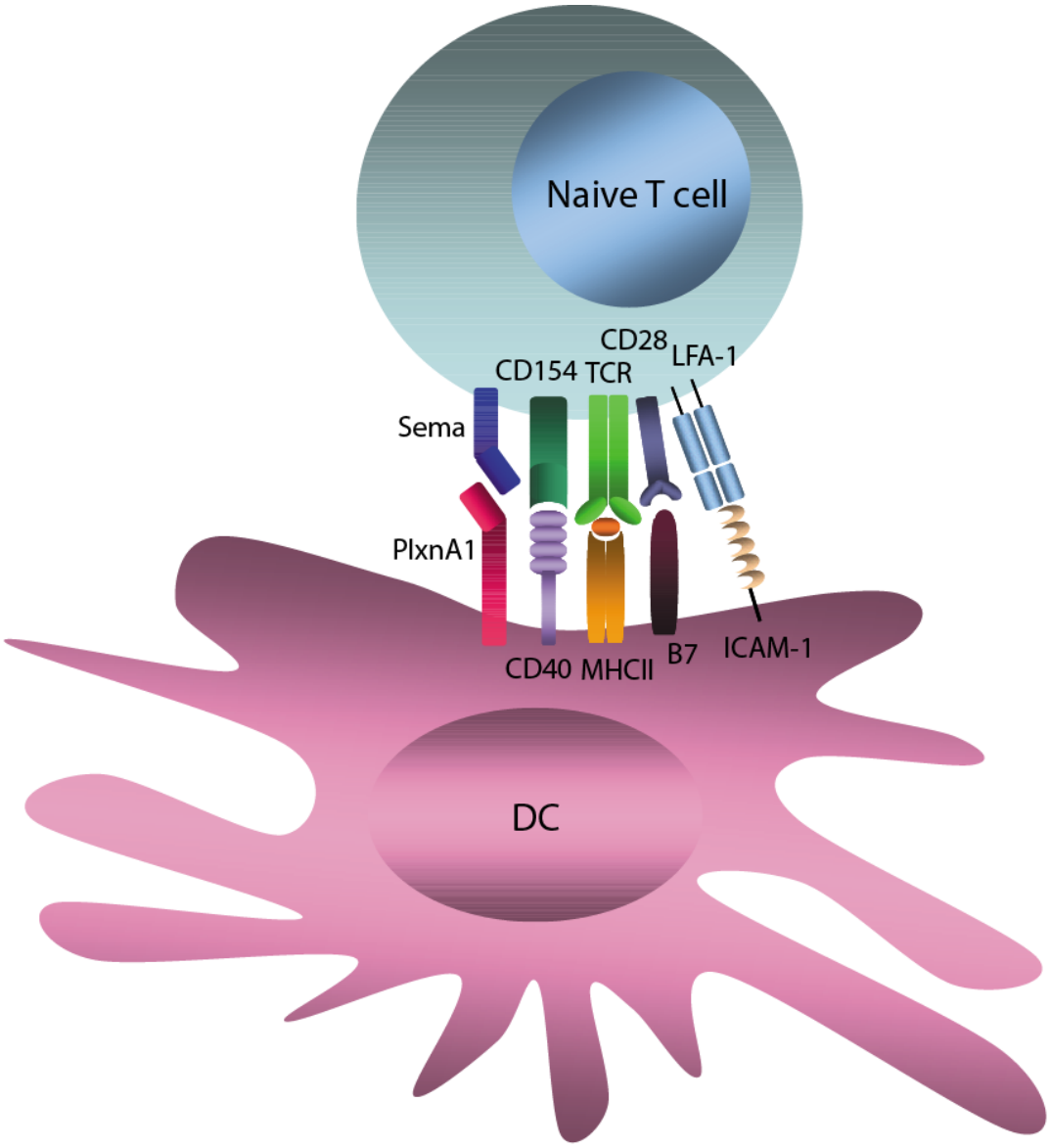


Figure 4.1. Diagram of intercellular molecular interactions on the surface of DCs and CD4$^+$ naïve T cells upon conjugation for T cell priming. Not only the interaction of antigen-loaded MHC class II molecules with cognate TCRs, but also other costimulatory interactions have been appreciated for their importance in T cell priming, such as the interaction of B7 with CD28, CD40 with CD40L (CD154), and ICAM-1 with LFA-1. PlxnA1 interaction with its ligand on T cells is now evidently an additional co-stimulation for T cell activation.

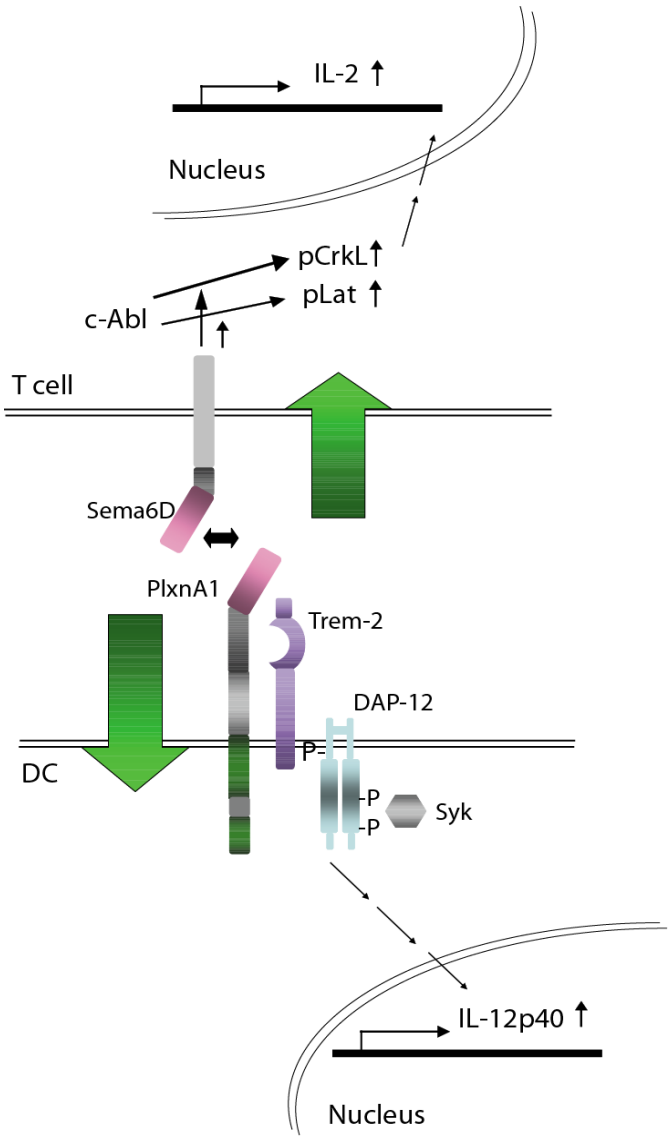


Figure 4.2. Diagram of Sema6D stimulation and subsequent signaling events upon DC-T cell interaction. It is a collective illustration of known signaling events upon Sema6D interaction with PlxnA1 upon DC-T cell interaction. In the late stage of T cell response, Sema6D stimulation is shown to activate c-Abl kinase activity in T cells, resulting in phosphorylation of Lat as well as Ckrl, leading to augmented IL-2 production [79]. On DC side, DAP12 is shown to be activated upon Sema6D, leading to elevated levels of IL-12 production, although PlxnA1 association with Trem-2-DAP12 complex does not seem to be changed by Sema6D engagement [29].

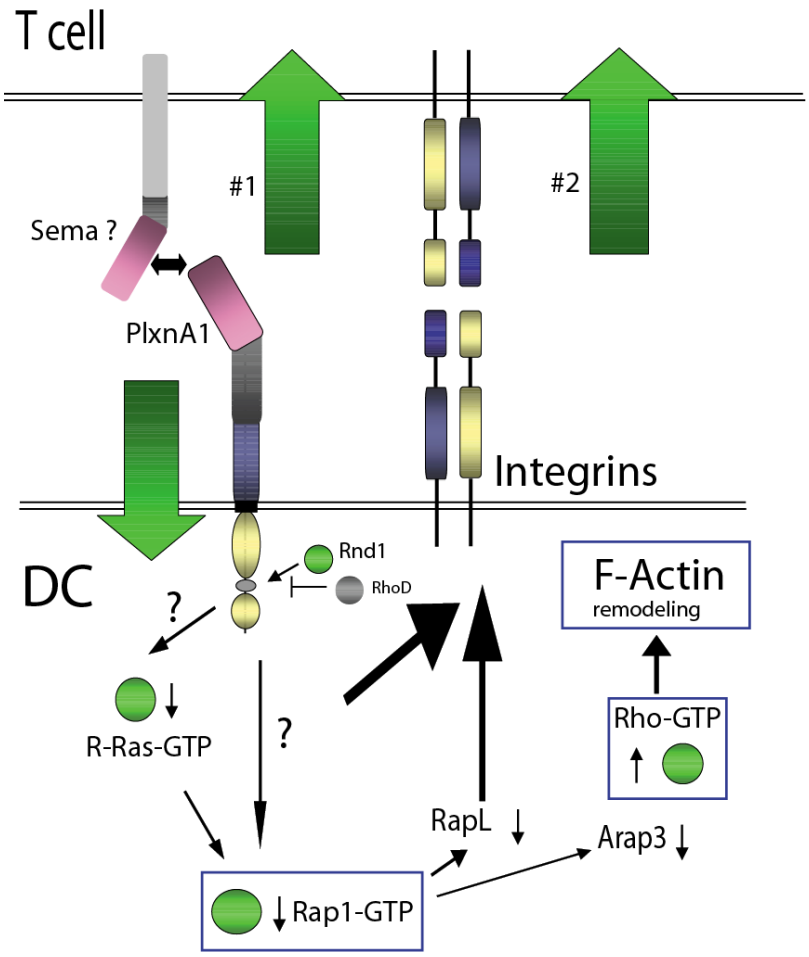


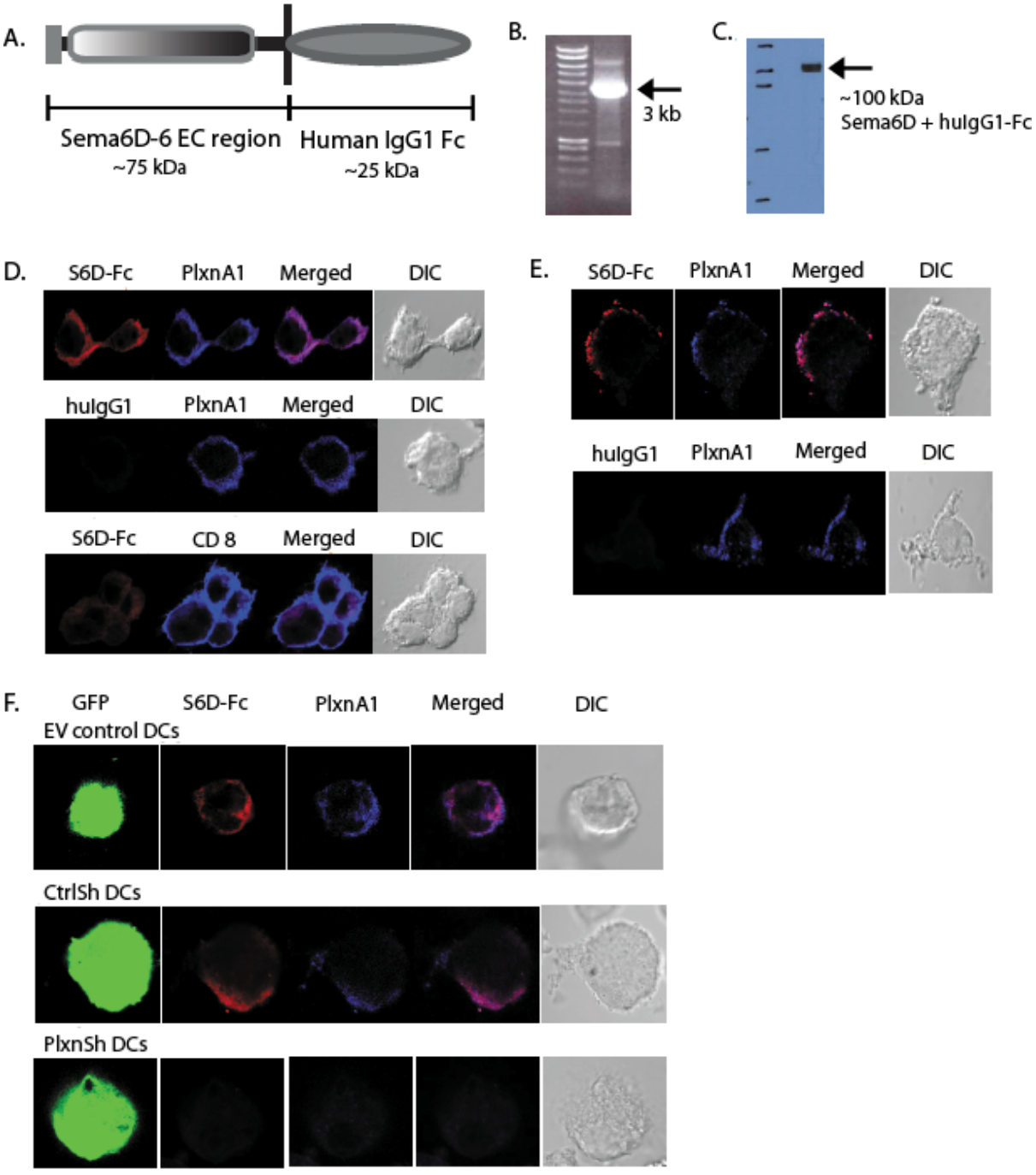
Figure 4.3. Diagram of hypothetical signaling events stimulated by PlxnA1 upon engagement with its ligand on naïve T cells in the context of DC-T cell conjugates. Upon T cell priming, PlxnA1 interaction with its ligand is thought to not only stimulate T cells directly (#1) but also stimulate DCs for further T cell stimulation (#2). Signaling events in DCs involving Rap1 possible regulation by PlxnA1 is expected to contribute to the latter (#2). Rap1 has been connected to suppression of Rho via its effector molecule, Arap3, a RhoGAP. In addition, Rho is activated upon T cell stimulation only in the presence of PlxnA1, as shown in Chapter II. It is also shown that Rap1 is activated by R-Ras. Thus, based on our preliminary data that a level of activated Rap1 in DCs upon T cell conjugation was elevated in the absence of PlxnA1 as shown in Chapter III, it is hypothesized that Rap1 is regulated by PlxnA1 either via its R-RasGAP activity or directly with its possible RapGAP activity. Rap1 is hypothesized not only to signal for actin remodeling through Rho, but also to signal for modulation of integrin activity through its effector molecules such as RapL, both of which are thought to contribute to optimal T cell response.

APPENDIX

Figure 5.1. Detection of Sema6D-Fc fusion protein colocalization with PlxnA1via confocal microscopy. The extracellular portion of Sema6D was fused with the Fc protion of human IgG1 in the pCDNA3 backbone (A). A 3 kb full-length cDNA encoding Sema6D isoform 6 (Sema6D-6) was isolated from mouse brain RNA by RT-PCR (B). Expression of Sema6D-Fc (100 kDa) from drug-selected Chinese hamster ovary stable cell lines was confirmed by western-blotting (C). Cos-7 cells were transfected with either a PlxnA1 or CD8α-expressing plasmid and then incubated with biotin-conjugated Sema6D-Fc fusion protein (S6D-Fc). Biotin-Sema6D-Fc was detected by streptavidin (SA)-conjugated Alexa Fluor 555 (red). PlxnA1 was detected by staining with anti-PlxnA1 rabbit polyclonal Ab followed by Alexa Fluor 647-conjugated anti-rabbit IgG (Blue). CD8α was detected with an anti-CD8α mAb followed by anti-mouse Alexa Fluor 647. Purple color demonstrates Ecolocalization of biotin-S6D-Fc with PlxnA1 (D). PlxnA1-expressing mouse DCs cultured for 12 days were incubated with biotin-S6D-Fc or control human IgG followed by the staining procedure described in D (E). To confirm that PlxnA1 expression by DCs was necessay for Sema6D-Fc association, we used a small hairpin RNA (ShRNA) to reduce PlxnA1 expression as described in Chapter II [78]. DCs transduced with an empty retroviral control (EV), a retrovirus containing a mutated shRNA (CtrlSh), or a retrovirus bearing shRNA for PlxnA1 (PlxnSh) were used in a localization assay with Sema6D-Fc. The staining protocol is identical to the one described in D. Purple color demonstrates that the red signal for biotin-S6D-Fc colocalized with the blue signal for PlxnA1. A reduction in PlxnA1 expression correlated with a reduced capacity of Sema6D-Fc to associate with DCs.

Published in O'Connor, B.P., et al. Semaphorin 6D regulates the late phase of $CD4^+$ T cell primary immune responses. 2008, *PNAS* 105(35): 13015-20.

Figure 5.1.


A.
Sema6D-6 EC region
~75 kDa
Human IgG1 Fc
~25 kDa
B.
3 kb
C.
~100 kDa
Sema6D + huIgG1-Fc
D.
S6D-Fc
PlxnA1
Merged
DIC
huIgG1
PlxnA1
Merged
DIC
S6D-Fc
CD 8
Merged
DIC
E.
S6D-Fc
PlxnA1
Merged
DIC
huIgG1
PlxnA1
Merged
DIC
F.
GFP
S6D-Fc
PlxnA1
Merged
DIC
EV control DCs
CtrlSh DCs
PlxnSh DCs

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