Cutting Edge: Rho Activation and Actin Polarization Are Dependent on Plexin-A1 in Dendritic Cells

So-Young Eun, Brian P. O'Connor, Athena W. Wong, Hendrick W. van Deventer, Debra J. Taxman, William Reed, Ping Li, Janice S. Blum, Karen P. McKinnon and Jenny P.-Y. Ting

*J Immunol* 2006; 177:4271-4275; doi: 10.4049/jimmunol.177.7.4271

http://www.jimmunol.org/content/177/7/4271

---

**Why The *JI***?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**  This article cites **33 articles**, 9 of which you can access for free at: http://www.jimmunol.org/content/177/7/4271.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cutting Edge: Rho Activation and Actin Polarization Are Dependent on Plexin-A1 in Dendritic Cells

So-Young Eun,† Brian P. O’Connor,‡ Athena W. Wong,§ Hendrick W. van Deventer,‡ Debra J. Taxman,‡ William Reed,‡ Ping Li,§ Janice S. Blum,§ Karen P. McKinnon,† and Jenny P.-Y. Ting†‡

We recently identified expression of the semaphorin receptor, plexin-A1, 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 plexin-A1 gene expression using small hairpin RNA (shRNA) in primary bone marrow-derived DCs. We show that plexin-A1 localizes within the cytoplasm of immature DCs, becomes membrane-associated, and is enriched at the immune synapse in mature DCs. Reducing plexin-A1 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-abolition of T cell activation. We propose a mechanism of adaptive immune regulation in which plexin-A1 controls Rho activation and actin cytoskeletal rearrangements in DCs that is associated with enhanced DC-T cell interactions. The Journal of Immunology, 2006, 177: 4271–4275.

D endritic cells (DCs) are the most potent type of APC, and the only APC capable of initiating primary immune responses via presentation of Ag in the context of MHC class II (MHC-II) molecules (1, 2). Following initial Ag 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, plexins, and neuropilins (3, 4). Semaphorin family proteins were first observed in the CNS where they mediate repulsive and attractive axon guidance cues during neural development (5, 6). Semaphorins are both secreted and membrane bound, and they are recognized by plexin family receptors (6–9). Plexins mediate cytoskeletal rearrangements that can result in either axon extension or retraction via interaction of their conserved C-terminal plexin domain with Rho family GTPases (10, 11). A generally held hypothesis is that developing neurons may use plexins to regulate their cytoskeleton and the formation of synapses. However, most studies have relied upon overexpression system in cell lines, whereas regulatory mechanisms of plexin-A1 (PlexA1) in primary cells, neuronal or otherwise, are underexplored.

In the immune system, semaphorins, plexins, and neuropilins regulate various activities, including regulation of T cell activation, B cell signaling, monocyte cytokine production, and regulation of DC migration (12–22). We previously demonstrated that the inhibition of PlexA1 expression in a transformed DC cell line reduced T cell activation (15, 23). However, the mechanism through which PlexA1 regulates primary bone marrow-derived DC interactions with T cells has remained largely unexplored. We report the novel investigation of PlexA1 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- to 12-wk-old C57BL/6 mice from The Jackson Laboratory. OT-II mice, which express the OVA_{323-339} specific TCR transgene on the C57BL/6 background, were obtained from Dr. M. 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 bone marrow DCs were isolated and cultured as described previously (24). T cells from OT-II mice were isolated from the spleen and purified by negative selection with T enrichment columns (R&D Systems).

*Curriculum in Genetics and Molecular Biology, †Department of Microbiology and Immunology and the Lineberger Comprehensive Cancer Center, and ‡Department of Pediatrics and Center for Environmental Medicine and Lung Biology, University of North Carolina, Chapel Hill NC 27599; and §Department of Microbiology and Immunology and the Lineberger Comprehensive Cancer Center, University of North Carolina, CB7295, Chapel Hill, NC 27599. E-mail address: panyun@med.unc.edu

1 This work was supported by National Institutes of Health Grant AI29564. J.P.-Y.T. is a Sandler Program in Asthma Research awardee, and B.P.O is an Irving Institute Postdoctoral Fellowship awardee.

2 S.-Y.E., B.P.O., and A.W.W. contributed equally to this work and should be considered co-first authors.

3 Address correspondence and reprint requests to Dr. Jenny P.-Y. Ting, Lineberger Comprehensive Cancer Center, University of North Carolina, CB7295, Chapel Hill, NC 27599. E-mail address: panyun@med.unc.edu

4 Abbreviations used in this paper: DC, dendritic cell; MHC-II, MHC class II; PFA, paraformaldehyde.
Conjugation assay, C3 treatment, and immunofluorescence confocal microscopy

DCs at day 12 were harvested and pulsed overnight with 50 μg/ml 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 pulsed down in microtubes by centrifugation at 1500 rpm for 10 min and incubated at 37°C for 45 min in a water bath. Following conjugation, 1 × 10^3 cells in 100 μl of PBS were added onto each poly-L-lysine-coated coverslip (BD Biosciences), fixed in 4% paraformaldehyde (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 Ab for 1 h at room temperature. For actin cytoskeleton, Alexa 647-conjugated phalloidin was used. Cover-slips 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 days 11.5 to 12 and during OVA-pulse for a total of 24 h. After washing, DCs were harvested and conjugated with OT-II T cells before confocal microscopy analysis. Phalloidin-AlexaFluor 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, PlexSh-, 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 ImageJ analysis. 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, PlexSh, and CtrlSh virus transduced DCs at days 10 and 12, as described previously (15). Staining was quantified with a BD FACSCalibur.

Assays for GTP-bound Rho, Rac, and Cdc42

Before 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 at 2°C for 20 min. Following conjugation, DCs and T cells were washed and harvested and subjected to costimulatory molecules (CD86: 97.3, 98.4, and 96.1% respectively) were not altered by viral transduction or the absence of PlexA1, reflecting specificity of the shRNA knockdown targeting (data not shown). In addition, DCs binding to an antigenic peptide (Eox) were also not altered by the shRNA (data not shown).

shRNA knockdown of PlexA1 inhibits DC mediated T cell activation

To functionally test the effect of retroviral PlexSh in primary mouse DCs, we performed an Ag presentation assay using whole OVA protein or OVA peptide-pulsed DCs and OT-II T cells. DCs transduced with PlexSh virus exhibited approximately a >80% reduction in T cell stimulation compared with DCs transduced with CtrlSh as assessed by IL-2 production (Fig. 2, A and B). Importantly, this reduction was observed with both whole OVA protein- and peptide-pulsed DCs (Fig. 2, A and B). These observations suggest that the inhibition of T cell

![Image](http://www.jimmunol.org)
activation associated with PlexA1 knockdown in DCs is not attributable to a defect in Ag processing or presentation by MHC-II.

Localization of PlexA1 in mature DCs at the immune synapse

To examine PlexA1 during DC maturation, DCs from C57BL/6 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 (15). Flow cytometry analysis of days 10 and 12 DCs revealed CD11c<sup>+</sup> 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-II when compared with day 10 counterparts (Fig. 3A). Immunofluorescent confocal microscopy was used to visualize the localization of PlexA1 during DC maturation. Although PlexA1 was absent in immature day 6 DCs, it was detected in maturing day 10 DCs as a cytoplasmic protein (Fig. 3B). Upon the addition of TNF-α, the protein became primarily located on the cell membrane, and this pattern was sustained at day 12 (Fig. 3B). As expected from previous reports, the MHC-II Ag (IA<sup>b</sup>) was detected as an intracellular protein in immature DCs but localized to the membrane periphery as the DCs matured (28). In day 12 DCs, PlexA1 was detected at the cell surface along with IA<sup>b</sup>, and the merged image suggests colocalization (Fig. 3C).

Given PlexA1’s localization to the cell membrane in mature DCs, we examined whether PlexA1 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 (29). Staining DC-T cell conjugates for ICAM-1, IA<sup>b</sup>, and TCR indicated that immune synapse-associated proteins of both DCs and T cells were enriched at the cell interface (Fig. 3). PlexA1 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<sup>b</sup>, ICAM-1, and PlexA1 staining demonstrates a nonpunctate 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-SMAD (Fig. 3D).

**FIGURE 3.** PlexA1 localizes to the DC surface and the DC-T cell interface. A. Flow cytometry analysis of DC phenotype at days 10 and 12 of in vitro culture, comparing CD11c, MHC-II, CD8, B220, CD14, and F4/80 expression to an appropriate isotype control. B. DCs were collected on days 6, 10, and 12 and stained for PlexA1 (green) and IA<sup>b</sup> (red) before resolving the images by confocal microscopy. C. Day 12 DCs were fixed and stained for PlexA1 (green) and IA<sup>b</sup> (red). D. 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 TCR-β (green) and IA<sup>b</sup> (red), second row ICAM-1 (green) and IA<sup>b</sup> (red), third row PlexA1 (green) and IA<sup>b</sup> (red), and bottom panels PlexA1 (green) and ICAM-1 (red). PlexA1 localized to the DC-T cell interface in 70 ± 4.2% of the 200 DC-T cell conjugates counted from five independent experiments. DIC, Differential interference contrast.
accumulation of active Rho, Rac, or Cdc42 (Fig. 4).

Given that plexins are known to regulate actin and cytoskeleton rearrangements, we examined the possibility that PlexA1 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, whereas DCs transduced with PlexSh virus show dispersed actin (Fig. 4A). The F-actin signals are mostly attributed to DCs as nonassociated and DC-associated T cells in these images do not exhibit much actin staining. Cells transduced with PlexSh or CtrlSh were selected based on GFP expression.

The intensity of actin staining at the interface of DC-T cell conjugates was quantified (Fig. 4B). Additionally, a double-blind study revealed that cell conjugates formed with PlexA1-deficient DCs displayed reduced actin polarization in >50% of DCs when compared with controls (Fig. 4C).

Given that small GTPases are known to regulate the actin cytoskeleton, we next examined the ability of PlexA1 to regulate Rho, Rac, or Cdc42 in DCs during interactions with T cells (32). OVA-pulsed PlexSh- or CtrlSh-transduced DCs were incubated with fixed OT-II T cells for 20 min before lysis. To analyze Rho activation from the DCs but not the T cells, OT-II T cells were fixed for 1 min with 4% PFA before incubation with DCs.

Fixed OT-II T cells did not exhibit any significant accumulation of active Rho, Rac, or Cdc42 (Fig. 4D, left panels). To assay for endogenous Rho, Rac, and Cdc42 activity in DCs, the mixed cultures were lysed and precipitated with RBP or PBD-bound agarose beads for GTP-bound Rho, Rac, or Cdc42, followed by immunoblotting (Fig. 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. 4D). The Rho family activation assay revealed two observations. First, efficient activation of Rho, Rac, and Cdc42 in DCs requires Ag-specific interaction of T cells with DCs (compare pulsed to unpulsed DC).

Second, inhibition of PlexA1 expression by shRNA in DCs results in decreased levels of GTP-Rho compared with DCs transduced with CtrlSh virus (compare pulsed DC). Significantly, PlexA1 inhibition affected Rho activation but not Rac or Cdc42 activation in pulsed DCs. These results illustrate that Ag-specific DC-T cell interactions are required for the activation of Rho, Rac, and Cdc42 and that PlexA1 regulates Rho activation in Ag-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. 4F). We also observed similar results with Y27632, an inhibitor of ROCK (Rho kinase, an effector molecule of RhoA) ROCK kinase (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. 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 PlexA1 expression is essential for optimal activation of T lymphocytes. PlexA1 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. 3) (23). We also report the novel finding of Rho activation and actin cytoskeleton regulation by a plexin in the immune system. It is clear from our observations that PlexA1 regulates Rho activation in DCs. Significantly, we observed that PlexA1 regulation of Rho activation was distinct from activation of Rac or Cdc-42 (Fig. 4D). In the absence of PlexA1 expression, we observed a significant loss of actin polarization, Rho activation, and T cell stimulation by DCs (Figs. 2 and 4). Previous studies have illustrated the importance of actin rearrangements at the immunological synapse for efficient T cell-DC interactions, and our data support these findings. We suggest a model in which PlexA1 regulates Rho activation and subsequent actin polarization in DCs. Our current work also demonstrates that PlexA1 expression is critical for DC-mediated activation of T lymphocytes in a manner distinct from MHC-II processing or altered expression of costimulatory molecules (Fig. 2). One likely scenario is that Rho activation via PlexA1 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. 4, E and F). In summary, this report begins to address the mechanism of DC-T cell regulation by PlexA1 expression at the immune synapse.

Note added in proof: The recently published report by Takegahara et al. (33) demonstrates the importance of PlexA1 in primary DCs ex vivo.

Acknowledgments

We thank Drs. Sergio Quezada and Steven Fiering for their help, Dr. Mick Croft for the OT-II mice, Drs. Neil Coffield and Lishan Su for the retroviral vector, pHSPG, Dr. Keith Burridge and Lisa Sharek for their generous gift of Croft for the OT-II mice, Drs. Neil Coffield and Lishan Su for the retroviral vector, pHSPG, Dr. Keith Burridge and Lisa Sharek for their generous gift of

Disclosures

The authors have no financial conflict of interest.

References


3. Dustin, M. L., and D. R. Colman. 2002. Neural and immunological synaptic rela-


7. 767– 811.


