CD2-Associated Protein Regulates Plasmacytoid Dendritic Cell Migration, but Is Dispensable for Their Development and Cytokine Production

Subhashini Srivatsan, Melissa Swiecki, Karel Otero, Marina Cella and Andrey S. Shaw

*J Immunol* 2013; 191:5933-5940; Prepublished online 11 November 2013; doi: 10.4049/jimmunol.1300454

http://www.jimmunol.org/content/191/12/5933
CD2-Associated Protein Regulates Plasmacytoid Dendritic Cell Migration, but Is Dispensable for Their Development and Cytokine Production

Subhashini Srivatsan,* Melissa Swiecki,* Karel Otero,*1 Marina Cella,* and Andrey S. Shaw*†‡

Plasmacytoid dendritic cells (pDCs) are a dendritic cell subset that secrete type I IFNs in response to microbial stimuli. The scaffold protein, CD2-associated protein (CD2AP), is a marker of human pDCs as it is highly expressed in this cell type. Recently, in human pDCs, decreased CD2AP expression appeared to enhance the production of type I IFNs via an inhibitory receptor-induced signaling cascade. In this study, we sought to determine the role of CD2AP in murine pDCs using CD2AP knockout (KO) mice. CD2AP was dispensable for the development of pDCs and for the upregulation of activation markers following stimulation. Loss of CD2AP expression did not affect the production of type I IFNs stimulated by TLR ligation, and only slightly impaired type I IFN production when inhibitory pathways were engaged in vitro. This was also confirmed by showing that CD2AP deficiency did not influence type I IFN production by pDCs in vivo. Because CD2AP plays a role in regulating actin dynamics, we examined the actin cytoskeleton in pDCs and found that activated CD2AP KO pDCs had significantly higher levels of actin polymerization than wild-type pDCs. Using two different inflammation models, we found that CD2AP KO pDCs have a defect in lymph node migration, correlating with the defects in actin dynamics. Our work excludes a role for CD2AP in the regulation of type I IFNs in pDCs, and suggests that the major function of CD2AP is on the actin cytoskeleton, affecting migration to local lymph nodes under conditions of inflammation. *The Journal of Immunology, 2013, 191: 5933–5940.

Plasmacytoid dendritic cells (pDCs) were originally identified as a rare subset of human peripheral blood leukocytes that secrete abundant amounts of type I IFNs in response to foreign nucleic acids (1, 2). Shortly after the characterization of human pDCs as a unique immune cell lineage, murine counterparts of human pDCs were identified and shown to share several key features of human pDCs (3–5). These cells express the microbial pattern recognition receptors, TLR7 and TLR9, which enable them to recognize ssRNA and unmethylated CpG-containing DNA sequences, respectively. Stimulation with natural or synthetic ligands of these TLRs leads to the production of type I IFNs, a major defining feature of this cell type, as well as other proinflammatory cytokines and chemokines (6, 7). Together, these cytokines and chemokines promote recruitment and/or activation of immune cells, including NK cells, T cells, and professional APCs (8–12). In addition, pDCs themselves have been shown to function as APCs (13, 14). In this manner, pDCs can function to bridge innate and adaptive immunity.

pDCs have also been implicated in the pathophysiology of a variety of human diseases, ranging from autoimmune disorders [systemic lupus erythematosus (10, 15), psoriasis (16, 17), Kikuchi’s disease (18)] to cancers [Castleman’s disease, Hodgkin’s lymphoma, hematodermic neoplasia; reviewed in (19)]. Until recently, there were few known lineage markers that could specifically identify pDCs in patient samples. Whereas BDCA-2, a type II C-type lectin, was reported to be a specific marker of pDCs (20), it is only expressed on a proportion of pDC neoplasias (21–23). In addition, problems may arise in cases in which the currently used markers are absent or aberrantly expressed, further emphasizing the need for additional pDC-specific markers.

CD2-associated protein (CD2AP) was identified as a novel, robust, and specific marker for human pDCs in steady state as well as in neoplastic conditions (24). CD2AP is a scaffold protein implicated in a variety of biological processes. CD2AP was originally identified as a molecule involved in organization of the specialized interface between a T cell and an APC called the immunological synapse (25). Presently, CD2AP is implicated to play a role in downregulation of receptor tyrosine kinases, in coordinating vesicular trafficking and degradation of ubiquitinated receptors, as well as in actin cytoskeleton remodeling (26–34).

The high expression of CD2AP in human pDCs led us to investigate whether CD2AP was also expressed in murine pDCs and whether CD2AP was important for pDC development and function. In this study, we found that CD2AP is also highly expressed in murine pDCs, but was dispensable for their development and for the secretion of TLR-induced type I IFNs and proinflammatory cytokines. However, primary CD2AP-deficient pDCs had alterations in actin polymerization levels, and, using two different models of inflammation-induced

*Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110; and †Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110
1Current address: Biogen Idec, Cambridge, MA.
Received for publication February 15, 2013. Accepted for publication October 8, 2013.
This work was supported by the Howard Hughes Medical Institute and National Institutes of Health Grants DK058366-13 and AI057966-09. S.S. was supported by a predoctoral fellowship from the Cancer Research Institute. M.S. is supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant 1K01DK095972-01A1. M.C. is supported by National Multiple Sclerosis Society Grant RG4687A1/1.
Address correspondence and reprint requests to Dr. Andrey S. Shaw, Washington University, 660 South Euclid Avenue, Campus Box 8118, St. Louis, MO 63110. E-mail address: shaw@pathology.wustl.edu
Abbreviations used in this article: BM, bone marrow; BMpDC, BM-derived plasmacytoid dendritic cell; BST2, BM stromal Ag 2; CD2AP, CD2-associated protein; Flt3L, Fms-like tyrosine kinase 3 ligand; KO, knockout; MCMV, murine CMV; MOI, multiplicity of infection; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cell; SiglecH, sialic acid–binding Ig-like lectin H; VSV, vesicular stomatitis virus; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/2013 $16.00.
www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300454
pDC recruitment, we also demonstrated defects in migration to lymph nodes. Thus, our data suggest that CD2AP has an essential role in regulating efficient migration of pDCs during inflammation.

Materials and Methods

Mice, Abs, and reagents

All animals were handled in accordance with the policies administered by the National Institutes of Health and the Washington University Institutional Animal Care and Use Committee. The CD2AP knockout (KO) mouse was previously generated in our laboratory (35). With the exception of siaclic acid–binding Ig-like lectin H (Siglec-H)-PE (eBiosciences), all other Abs to mouse proteins were purchased from BioLegend. Mouse Fms-like tyrosine kinase 3 ligand (Flt3L), CXCL12, and CCL19 were purchased from PeproTech. CpG-A oligodeoxynucleotide (ODN) 2216 was purchased from Operon. Resiquimid was purchased from Invivogen.

Generation of bone marrow chimeras

Bone marrow (BM) was extracted from 2-wk-old, nonproteinuric, wild-type (WT), or CD2AP KO mice, which were congenically marked by the expression of the cell surface marker, CD45.2. BM cells were depleted of RBCs, resuspended in PBS, and counted. Recipient CD45.1+ mice were irradiated at 1000 rad either 1 d prior to or 6–8 h prior to receiving donor BM. Irradiated recipients were anesthetized, and the BM was delivered to each mouse via i.v. injections of 100 μl containing 5–10 million cells. The mice were given antibiotics in their water over a period of 2 wk, by which time their immune system should be reconstituted by donor BM.

Isolation of primary mouse pDCs

Mouse femurs were flushed to obtain BM, which was subjected to a Histopaque (25), followed by secondary goat anti-rabbit AF647 (1:10,000 dilution). Slides were mounted for microscopy. Images were acquired using an Olympus Confocal Microscope FV1000, and image analysis was performed using Fluoview software.

In vitro migration assay

A total of $1 \times 10^6$ BM cells or BM-pDCs in 100 μl was loaded into transwells (Corning BV; 5 μm pore size) that were placed in 24-well plates containing 400 μl medium only or medium supplemented with various concentrations of CCL19 or CXCL12 (PeproTech). After a 3-h incubation at 37°C, the cells that had migrated into the bottom chamber were collected, mixed with a defined number of Calibrate beads (BD Biosciences), and stained with B220-allophycocyanin and Siglec-H-PE to detect pDCs. The cell–bead mixture was then subjected to analysis by flow cytometry for cell enumeration.

Induction of lymph node inflammation and in vivo homing assay

For the in vivo homing assay using Mycobacterium tuberculosis (Difco Laboratories), mice were injected with heat-killed M. tuberculosis in their left hind footpad and leg (500 μg/injection) at 72 and 24 h prior to excising the local lymph nodes for analysis. For the viral in vivo homing assay, mice were injected with $1 \times 10^6$ PFU vesicular stomatitis virus (VSV) in their left hind footpad at 72 h prior to excising the local lymph nodes for analysis. In both homing assays, contralateral and draining popliteal lymph nodes were analyzed.

Western blotting

BM-sorted primary pDCs as well as WT pDCs were lysed in 1% Nonidet P-40 lysis buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA [pH 7.6]) with protease inhibitors. Lysates were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and blocked overnight in 5% BSA in 1× TBS buffer (0.1 M Tris HCl, 1.5 M NaCl, 0.5% Tween 20 [pH 7.5]). CD2AP and actin expression were detected using anti-CD2AP (1:10,000 dilution) and anti-β-actin (1:5,000 dilution; Sigma-Aldrich) primary Abs and Li-Cor anti-rabbit and anti-mouse secondary Abs. Membranes were scanned and analyzed using a Li-Cor imaging system (Lincoln, NE).

In vitro preparation of BM-derived pDCs

To prepare BM-derived pDCs (BM-pDCs), mouse BM was depleted of RBCs and resuspended at $1.5 \times 10^6$ cells/ml in complete RPMI 1640 medium, supplemented with 10 ng/ml Flt3L. On day 3, 1 ml medium was replaced with fresh medium, and an additional 1 ml medium was added on day 6. The cells were used for experiments on day 7.

Assessment of cytokine production

Primary pDCs sorted from BM were counted and plated at 40,000–100,000 cells/well in a 96-well plate. For the Ab cross-linking experiments, the 96-well plates were precoated overnight with the following Abs at 10 μg/ml in PBS: anti-CD16/32, anti-BM stromal Ag 2 (B2T2), anti-Siglec-H, or rat IgG2b (isotype control). The cells were stimulated in vitro for 24 h with CpG ODN 2216 (6 μg/ml). For in vitro stimulation with murine CMV (MCMV), primary pDCs were treated with MCMV tissue culture stocks (37) at different multiplicities of infection (MOIs). For in vivo analysis of type I IFN production, 8-wk-old BM chimeras were injected i.v. with 6 μg CpG-A ODN 2216 complexed with 30 μl dioleoyl-trimethylammonium-methyl sulphate (Roche) in PBS, and serum was collected 8 h postinjection. IFN-α released into culture supernatants was measured by ELISA (PBL IFN Source). Inflammatory cytokines in the supernatants were assayed by cytometric bead array (BD Biosciences).

Immunofluorescence

BM-sorted primary pDCs were plated overnight on fibronectin-coated (10 μg/ml; Life Technologies/Invitrogen, Grand Island, NY) glass-bottom plates and either left untreated or stimulated with Resiquimid overnight. The next day, the activated cells were either treated or not with a mixture of chemokines (100 ng/ml CCL19 and 100 ng/ml CXCL12) for 45 min and then fixed in 3.8% paraformaldehyde. The fixed cells were then permeabilized in 0.1% Triton X-100 in PBS and stained with rhodamine-conjugated phalloidin (1:1000 dilution; Invitrogen) and Hoechst (1:300 dilution). Slides were mounted for microscopy. Images were acquired using an Olympus Confocal Microscope FV1000, and image analysis was performed using Fluoview software.

FACS analysis

Organs were analyzed after RBC lysis and Fc blocking (anti-CD16/CD32). For intracellular staining for CD2AP, the cells were fixed with 3.8% paraformaldehyde, permeabilized, and stained with anti-CD2AP [1:10,000 dilution (25)], followed by secondary goat anti-rabbit AF647 (1:10,000 dilution; Invitrogen) in FACS permeabilization/staining buffer (0.05% saponin and 2% FCS in PBS). pDCs were stained with B220-allophycocyanin and Siglec-H-PE or B220-allophycocyanin and CD11c-FC (PeproTech); for BM chimeras, additional staining was performed with CD45.1-PerCPCy5.5 and CD45.2-allophycocyanin Cy7. Flow cytometry was conducted on BD LSR II and BD FACSAria II flow cytometers, and the data were analyzed using FlowJo software (Tree Star).

Statistical analysis

The statistical significance of differences in mean values was analyzed with the unpaired, two-tailed Student t test. The p values < 0.05 were considered statistically significant.

Results

CD2AP is highly expressed in mouse pDCs

Human pDCs have been shown to express high levels of CD2AP (24, 38). To confirm CD2AP expression in mouse pDCs, we sorted primary pDCs directly from BM of WT B6 mice and immunoblotted pDC lysates with an anti-CD2AP Ab (Fig. 1A). We observed similar expression of CD2AP in mouse pDCs compared with mouse podocytes, which express high levels of CD2AP. In addition, we examined CD2AP expression in splenic immune cell subsets by intracellular staining. These data verified CD2AP expression in pDCs and also demonstrated that pDCs express higher levels of CD2AP compared with other major immune cell populations in the spleen.
CD2AP is highly expressed in murine pDCs. (A) Murine pDCs were gated on B220<sup>+</sup>Siglec<sup>H</sup><sup>+</sup> cells (not depicted) and sorted for immunoblotting. Mouse podocytes were used as a positive control, and both cell types were blotted for CD2AP expression using an anti-mouse CD2AP Ab. Lane 1, 0.5 × 10<sup>6</sup> pDCs; lane 2, m.w. marker; and lane 3, 0.5 × 10<sup>6</sup> podocytes. (B) Intracellular FACS analysis for CD2AP expression in the major immune cell subsets of the mouse spleen. Lower panel, Shows a key of the lymphoid (right column) and myeloid cells (left column) examined.

FIGURE 1.

CD2AP is dispensable for pDC development

We first decided to assess whether CD2AP is required for pDC development because CD2AP has previously been shown to be a transcriptional target of E2-2, the master transcription factor regulating pDC development (39). The CD2AP KO mouse develops proteinuria at 3 wk of age and succumbs to renal failure by 6 wk of age (35, 40). Therefore, we chose to study 2.5-wk-old, nonproteinuric CD2AP KO mice. Analysis of pDC populations in the BM, spleen, thymus, and lymph nodes of WT and CD2AP KO mice showed no differences in frequencies as well as total numbers of pDCs (Fig. 2A–D and data not shown). Interestingly, parallel studies conducted in 5-wk-old proteinuric CD2AP KO mice demonstrated significantly reduced pDC frequencies and numbers, suggesting that kidney failure negatively impacts pDC numbers in lymphoid organs and in the circulation (Fig. 2 and data not shown). To verify that CD2AP is not required for pDC development, we generated BM chimeras using BM from 2.5-wk-old WT or CD2AP KO donors and reconstituted WT irradiated recipients. This allowed us to study the impact of CD2AP deficiency specifically in the hematopoietic compartment without the complications of renal failure. We obtained chimeras with ≈90% chimerism (as determined by CD45.1 and CD45.2 staining; data not shown). As expected, analysis of the BM, spleen, peripheral lymph nodes, thymus, liver, and gut intraepithelial lymphocytes, 8 wk postreconstitution, revealed no defects in pDC development (Fig. 3A, 3B).

FIGURE 2.

Proteinuric CD2AP KO mice exhibit defects in pDC frequencies and numbers. (A) FACS profiles of pDC development of WT and CD2AP KO mice aged 2.5 wk (left two panels, nonproteinuric) or 5 wk (right two panels, proteinuric). Frequencies of BM pDCs (B) and splenic pDCs (C) and total numbers of splenic pDCs (D) for both age groups of animals (***p = 0.002, ****p < 0.0001). pDCs were identified by Siglec<sup>H</sup>B220<sup>+</sup> expression. Black columns indicate WT samples and white columns are KO samples. Four mice were used per group.
the chimeras was also unaffected by CD2AP deficiency (Fig. 3C). Altogether, these data show that CD2AP is not required for pDC development in mice.

**CD2AP deficiency in pDCs does not affect the expression of activation markers**

To determine whether TLR-induced maturation of pDCs was affected by the absence of CD2AP, we first examined various activation markers at steady state in BM and splenic pDCs, and found them to be normal (Fig. 4A). Next, we isolated BM from non-proteinuric 2-wk-old WT and CD2AP KO mice and cultured it in the presence of Flt3L to generate BMpDCs. Both WT and CD2AP KO BM produced BMpDCs at similar frequencies (Fig. 4B). Next, we examined the upregulation of activation markers by BMpDCs in response to stimulation with Resiquimod, a TLR7 agonist, (Fig. 4C), and CpG-A, a TLR9 agonist (data not shown). The stimulated CD2AP KO BMpDCs did not exhibit any defect in their ability to upregulate MHC II, MHC I, CD80, CD86, and CCR7 and to downregulate CD62L (Fig. 4C). Taken together, our data demonstrate that CD2AP does not play any role in regulating the expression of activation markers in murine pDCs.

**CD2AP deficiency in pDCs does not affect the production of type I IFNs and inflammatory cytokines**

A prominent feature of pDCs is their ability to secrete large amounts of type I IFNs and inflammatory cytokines, such as TNF-α, in response to viral or bacterial TLR ligands. Recently, it was reported, using a human pDC cell line, that short hairpin RNA-mediated inhibition of CD2AP expression attenuated the production of type I IFNs, downstream of CpG-A stimulation prior to cross-linking with the inhibitory receptor complex, BDCA2/FcεRIγ (38). To determine whether CD2AP plays a similar role in murine pDCs, we sorted primary pDCs from the BM of WT and CD2AP KO chimeric mice, and stimulated them with CpG-A in the presence or absence of plate-bound Abs against receptors such as BST2 and SiglecH. Cross-linking either of these receptors has an inhibitory effect on type I IFN production in pDCs (41–43). Additionally, we used anti-Cd16/32 to mimic cross-linking with FcγRI (38) and rat IgG2b as an isotype control. Stimulation with CpG-A alone or after cross-linking with anti-Cd16/32 did not reveal any defects in production of IFN-α or TNF-α by CD2AP KO pDCs (Fig. 5A, 5B). However, when either BST2 or SiglecH was cross-linked prior to CpG-A stimulation, we noted a mild and reproducible defect in type I IFN and TNF-α production in CD2AP KO pDCs relative to WT pDCs (Fig. 5A, 5B).

Next, we measured serum type I IFN and inflammatory cytokine levels, 8 h after an i.v. injection of CpG-A. Because the type I IFN response to CpG-A is mediated by pDCs, serum IFN-α levels at this early time point should be a good reflection of cytokine production by pDCs. Our in vivo results revealed that there are no significant defects in IFN-α or TNF-α production in CD2AP KO chimeric mice (Fig. 5C, 5D).

Finally, we examined cytokine production by pDCs in response to MCMV in vitro (Fig. 5E, 5F). WT and CD2AP KO pDCs were infected with MCMV at three different MOIs, which revealed no differences in the production of type I IFN and TNF-α. Taken together, these data suggest that, in murine pDCs, CD2AP does not play a physiological role in regulating cytokine production.

**CD2AP affects actin polymerization in pDCs and is essential for pDC migration**

CD2AP has been implicated in playing a role in regulation of the actin cytoskeleton in podocytes (34, 44, 45). To examine the actin cytoskeletal organization in pDCs, primary WT and CD2AP KO pDCs were plated onto fibronectin-coated dishes and left untreated or were treated with Resiquimod overnight. The following day, the activated pDCs were briefly treated or not with a chemokine mixture of CCL19 and CXCL12, prior to staining with phalloidin. We found that whereas untreated CD2AP-deficient pDCs had no differences in actin polymerization compared with their WT counterparts, activated and

---

**FIGURE 3.** CD2AP is dispensable for pDC development. (A) Representative FACS plots of pDCs in the BM, spleen, and peripheral (axillary) lymph nodes in 8-wk-old CD2AP WT and KO BM chimeras. pDCs were stained with anti-B220 and anti-SiglecH. (B) Total number of pDCs in the organs indicated. (C) Total number of B cells in the organs indicated. Black columns indicate WT samples, and white columns are KO samples. ax LNs, Axillary lymph nodes; IELs, gut intraepithelial lymphocytes; ing LNs, inguinal lymph nodes; pop LNs, popliteal lymph nodes. Six to twelve chimeric mice were used per group.
chemokine-exposed KO pDCs had significantly higher actin polymerization levels (Fig. 6A). We also noted that CD2AP KO pDCs formed more exaggerated lamellipodia relative to the WT pDCs (Fig. 6B).

Because the regulation of the actin cytoskeleton is important for cell migration [reviewed in (46)], we tested whether CD2AP deficiency affected pDC migration. We examined in vitro migration to CCL19 or CXCL12 using primary pDCs as well as BMpDCs and observed no defects (Fig. 6C, 6D). Because in vitro migration assays may not be representative of migration through the three-dimensional architecture of tissue, we also examined migration in vivo. It is well established that pDCs are more abundant in inflamed lymph nodes of human patients with autoimmune diseases or infections compared to normal lymph nodes (1). Similarly, in mice, delivery of an inoculum...
of bacterial products to peripheral tissues results in the recruitment of pDCs to local lymph nodes. WT and CD2AP KO pDCs sorted from the BM were spread on fibronectin-coated plates and left untreated or stimulated with Resiquimod overnight. The next day, Resiquimod-activated pDCs were either left untreated or were stimulated for 45 min with CXCL12 and CCL19 (100 ng/ml). All cells were fixed and stained with phalloidin and Hoechst. (A) Normalized intensity of actin polymerization (average phalloidin intensity normalized to corresponding cell’s average Hoechst intensity) for all three conditions tested (untreated, Resiquimod treated, and Resiquimod and chemokine treated). Data are representative of two experiments with a total of 50–65 cells analyzed per condition. (B) Representative images of Resiquimod-activated WT (upper three panels) and CD2AP KO (lower three panels) pDCs showing the exaggerated lamellipodia formed in the KO cells. Phalloidin (red) and Hoechst (blue). Primary pDCs sorted from the BM (C) and BMpDCs (D) were mobilized in vitro to response to CCL19 (100 ng/ml) and CXCL12 (30 and 100 ng/ml). (E) Schematic of the inflammation model used to stimulate pDC migration to lymph node. For the M. tuberculosis model, mice were injected on days 0 and 2 with heat-killed M. tuberculosis applied to the left footpad and hindleg; right legs were left uninfected. For the VSV model, mice were injected on day 0 with 1 × 10^6 PFU applied to the left footpad; right footpads were left uninfected. On day 3, both contralateral and draining popliteal nodes were isolated for FACS analysis. (F) Numbers of pDCs that migrated into the contralateral and draining popliteal lymph nodes 72 h after M. tuberculosis or VSV treatment. Twelve to fourteen mice per group were used for the M. tuberculosis recruitment assay, and 6 mice per group were used for the VSV assay.

FIGURE 6. CD2AP plays a critical role in inflammation-induced migration of pDCs to local lymph nodes. WT and CD2AP KO pDCs sorted from the BM were spread on fibronectin-coated plates and left untreated or stimulated with Resiquimod overnight. The next day, Resiquimod-activated pDCs were either left untreated or were stimulated for 45 min with CXCL12 and CCL19 (100 ng/ml). All cells were fixed and stained with phalloidin and Hoechst. (A) Normalized intensity of actin polymerization (average phalloidin intensity normalized to corresponding cell’s average Hoechst intensity) for all three conditions tested (untreated, Resiquimod treated, and Resiquimod and chemokine treated). Data are representative of two experiments with a total of 50–65 cells analyzed per condition. (B) Representative images of Resiquimod-activated WT (upper three panels) and CD2AP KO (lower three panels) pDCs showing the exaggerated lamellipodia formed in the KO cells. Phalloidin (red) and Hoechst (blue). Primary pDCs sorted from the BM (C) and BMpDCs (D) were mobilized in vitro to response to CCL19 (100 ng/ml) and CXCL12 (30 and 100 ng/ml). (E) Schematic of the inflammation model used to stimulate pDC migration to lymph node. For the M. tuberculosis model, mice were injected on days 0 and 2 with heat-killed M. tuberculosis applied to the left footpad and hindleg; right legs were left uninfected. For the VSV model, mice were injected on day 0 with 1 × 10^6 PFU applied to the left footpad; right footpads were left uninfected. On day 3, both contralateral and draining popliteal nodes were isolated for FACS analysis. (F) Numbers of pDCs that migrated into the contralateral and draining popliteal lymph nodes 72 h after M. tuberculosis or VSV treatment. Twelve to fourteen mice per group were used for the M. tuberculosis recruitment assay, and 6 mice per group were used for the VSV assay.

of bacterial products to peripheral tissues results in the recruitment of pDCs to local draining lymph nodes (42, 47). Heat-killed M. tuberculosis was administered into the left hind footpads of WT and CD2AP KO BM chimeric mice in two successive inoculations on days 0 and 2 (Fig. 6E). On day 3, popliteal lymph nodes were analyzed from both the inoculated and the contralateral, uninjected legs. Compared with WT chimeric mice, the number of pDCs recruited to the inflamed lymph nodes of CD2AP KO mice was greatly reduced (Fig. 6F, p = 0.03). pDC recruitment to the contralateral popliteal node was also decreased in the KO chimeras (Fig. 6F, p = 0.06), implying that the condition under which the M. tuberculosis inoculum was administered induced a systemic reaction. Because steady-state popliteal node pDC numbers are the same in WT and KO chimeric mice (Fig. 1B), these data suggest that pDC recruitment to lymph nodes is impaired in the CD2AP KO.

Next, we examined a second stimulus to elicit pDC recruitment, VSV. VSV was administered as a single dose at day 0 to the footpad of WT and KO chimeric mice, and popliteal lymph nodes were analyzed on day 3 (Fig. 6E). Whereas there was no significant difference in pDC recruitment to the KO versus WT draining lymph node, there was a moderate decrease in pDC recruitment to the contralateral popliteal node in the KO (Fig. 6F, p = 0.06). We suspect that the robustness of the inflammatory response evoked by VSV infection might have overcome the CD2AP defect in pDC recruitment to infected draining lymph nodes. In more distal lymph nodes, in which the recruitment signals are weaker, the migration defect was more apparent.

We noted that in both infection models, the total number of cells in the lymph nodes from the KO chimeras was lower in both infected and contralateral lymph nodes (data not shown). This was especially apparent after VSV infection, in which a significant decrease in B cells was noted in both the infected and contralateral lymph nodes (data not shown). After M. tuberculosis infection, we observed a similar decrease in B cell recruitment to the contralateral lymph nodes of CD2AP KO chimeras (data not shown). These data suggest a novel role for CD2AP in regulating B cell migration during inflammation and imply that a broader defect in leukocyte recruitment to the lymph node may exist.
Altogether, our data demonstrate an important role for CD2AP in the migration of pDCs, and other leukocytes, to local lymph nodes during inflammation, and this is likely caused by defective actin dynamics.

**Discussion**

Previous reports demonstrate that CD2AP is highly expressed in both primary human pDCs (24), as well as in a pDC cell line derived from a leukemic patient (38). In this study, we report that CD2AP is also robustly expressed in murine pDCs. Indeed, relative to other major immune cell populations (conventional DCs, macrophages, T and B cells) examined in the spleen, we found that pDCs are among the highest expressers of CD2AP. In addition, whereas CD2AP was previously thought to be highly expressed in lymphoid cells (25), our work suggests that myeloid cells express higher relative levels.

CD2AP KO mice develop severe proteinuria after 4 wk of age and die of renal failure by 6 wk of age (40). Therefore, we initially studied development in younger, nonproteinuric CD2AP KO mice; no defects in pDC development were detected in these animals. Interestingly, we found that pDC numbers were significantly lower in proteinuric mice, suggesting that renal failure could have a negative impact on pDC development or survival. Because pDC frequencies and numbers can vary considerably between strains (48) and among different age groups within the same strain, we also examined older adult mice. Typically, the immune system is characterized in animals aged 6–8 wk, as this is the age at which the development of the mouse adult immune system is complete. Because CD2AP KO mice are in renal failure and close to death by this age, we generated BM chimeras derived from both WT and KO animals. BM chimeric animals were analyzed 8 wk after reconstitution, and no defects in pDC development were observed. Thus, extensive analysis of CD2AP KO mice revealed that CD2AP does not play a major role in pDC development.

Several reports emphasize the importance of pDCs in bridging the innate and adaptive immune systems during an infection. To communicate with other immune cells, pDCs upregulate activation markers and produce a wide range of cytokines and chemokines. We analyzed both pDCs extracted directly from the mouse as well as BMpDCs. There were no detectable defects in expression or upregulation of various markers with or without pDC activation using TLR agonists.

A major defining feature of pDCs is their ability to produce large quantities of type I IFNs. Recently, downregulation of CD2AP expression was shown to enhance the production of type I IFNs after inhibitory receptor engagement in a human pDC cell line (38). We tested this in primary pDCs from CD2AP-deficient mice and found that engagement of inhibitory pathways after TLR stimulation resulted in slightly attenuated production of type I IFNs in contrast to the increase reported in the human cell line. We detected no differences in type I IFN production after treatment with varying doses of Cpg-A or MCMV. More importantly, we saw no defects in cytokine production in CD2AP KO chimeric mice injected with the synthetic TLR9 ligand Cpg-A. Our results could be due to species-specific differences between humans and mice or that the leukemic pDC cell line used in the previous study may not accurately reflect regulation of type I IFN production in primary pDCs. We conclude that, at least in mice, the immunostimulatory role of pDCs as measured by cytokine production and activation marker expression/upregulation is not greatly influenced by CD2AP.

CD2AP has been previously implicated in actin cytoskeleton regulation by associating with actin-binding proteins (32, 33), being localized to actin-rich areas (31, 49, 50) and reported to interact directly with actin (45, 51, 52). We found, using phalloidin staining, striking differences between activated WT and KO cells. Activated CD2AP-deficient pDCs had higher levels of actin polymerization and formed more exaggerated lamellipodia compared with WT pDCs. This suggests that CD2AP may play a role in the regulation of actin polymerization at the cell periphery.

Because CD2AP is required for efficient cell migration (34), we analyzed whether CD2AP played a role in pDC migration in vitro and in vivo. In vitro transwell assays revealed no differences in pDC migration to either CCL19 or CXCL12. The normal absolute numbers of pDCs in CD2AP KO chimeric animals suggested that migration in steady state was normal. We therefore examined in vivo migration under conditions of inflammation. Inflammation induces pDCs to migrate to local lymph nodes (42, 47). CD2AP KO pDCs exhibited highly attenuated recruitment to both draining and contralateral lymph nodes in response to heat-killed M. tuberculosis. When a stronger, more physiological, viral stimulus was used to elicit pDC recruitment to the lymph nodes, no defect in pDC recruitment to the draining lymph node was seen. However, we did note a decrease in the number of pDCs in the contralateral lymph node. We suspect that the defect in migration of CD2AP-deficient pDCs was masked under conditions of strong inflammation. At distant lymph nodes, and/or where the inflammatory stimulus is weaker, the migration defect was more apparent.

In addition to the migration defect we observed in pDCs, we also noted a broader defect in leukocyte recruitment to the lymph nodes, suggesting a role for CD2AP in governing migration of other immune cells. B cells, which also express high levels of CD2AP, were found to have a migration defect to lymph nodes during viral infection. This finding illustrates the importance of CD2AP in regulating cell migration and a novel role for CD2AP that remains to be further characterized.

To our knowledge, these findings provide the first evidence that CD2AP is highly expressed in murine pDCs. Similar to humans, it appears that pDCs express CD2AP at some of the highest levels in the body and could therefore be used as a pDC-specific marker. Given their high level of expression, it was surprising that we could not detect a role for CD2AP in pDC development or in cytokine production. The CD2AP ortholog, CIN85, which is also expressed in murine pDCs (data not shown), may function to ameliorate the loss of CD2AP. Nonetheless, we did detect a defect in actin polymerization and migration. We suspect that high-level CD2AP expression must therefore indicate a specialized actin structure or specific regulatory structure that is present in pDCs. Future studies will provide insight as to why CD2AP is so highly expressed in pDCs and how it functions to regulate their actin cytoskeletal dynamics.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


