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A Critical Role for Prostaglandin E2 in Podosome Dissolution and Induction of High-Speed Migration during Dendritic Cell Maturation

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Dendritic cells (DCs) are professional APCs of the immune system that play a key role in regulating T cell-based immunity. The capacity of DCs to activate T cells depends on their maturation state as well as their ability to migrate to the T cell areas of draining lymph nodes. In this study, we investigated the effects of DC maturation stimuli on the actin cytoskeleton and integrin-dependent adhesion and migration. Podosomes, specialized adhesion structures found in immature monocyte-derived DCs as well as myeloid DCs, rapidly dissolve in response to maturation stimuli such as TNF-α and PGE2, whereas the TLR agonist LPS induces podosome disassembly only after a long lag time. We demonstrate that LPS-mediated podosome disassembly as well as the onset of high-speed DC migration are dependent on the production of PGs by the DCs. Moreover, both of these processes are inhibited by Ab-induced activation of β1 integrins. Together, these results show that maturation-induced podosome dissolution and loss of α5β1 integrin activity allow human DCs to undergo the transition from an adhesive to a highly migratory phenotype. The Journal of Immunology, 2006, 177: 1567−1574.

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Abbreviations used in this paper: DC, dendritic cell; ECM, extracellular matrix; iDC, immature DC; MCM, monocyte-conditioned medium; mDC, mature DC; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin.
such as PGE$_2$, produced by DCs during maturation, mediate podosome disassembly as well as the onset of high-speed migration.

Materials and Methods

Chemicals and Abs

The following Abs were used: anti-HLA class I (W6/32), anti-HLA-DR/DP (Q5/13), anti-CD80 (all from BD Biosciences), anti-CD14 and anti-CD83 (both from Beckman Coulter), anti-CD86 (BD Pharmingen), anti-CD209/DC-specific ICAM-3-grabbing nonintegrin (SIGN) (AZN-D1) (22), anti-$\beta$ integrin-activating epitope (TS2/16) (23), anti-$\beta$ integrin-blocking epitope (AILIB2) (Developmental Studies Hybridoma Bank), anti-$\beta$ integrin recognizing an active conformation (1G10) (24) (a gift from M. Humphries, Faculty of Life Sciences, University of Manchester, Manchester, U.K.), anti-$\alpha$ integrin (SAM-1), anti-vinculin (Sigma-Aldrich), mlgG1 (BD Biosciences), mlgG2a, mlgG2b, and rlgG1 isotype (BD Pharmingen). Alexa Fluor 488-labeled secondary Abs were from Molecular Probes (Molecular Probes), and FITC-labeled secondary Abs were from Zymed Laboratories and MP Biomedicals. Texas Red-conjugated phalloidin (Molecular Probes) was used to stain F-actin. Indomethacin was obtained from Sigma-Aldrich.

Preparation of DCs

DCs were generated from PBMCs, as described previously (25, 26). Monoocytes were derived from buffy coats or from a leukapheresis product. Plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhitaker). Cells were supplemented with 2% pooled human serum (PAA Laboratories, IL-4 (500 U/ml), and GM-CSF (800 U/ml) (both obtained from Schering-Plough International) (27), or in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% (v/v) FCS (Greiner Bioscience). Cells were incubated for 3 days and then transferred to fibronectin-coated plates (3g/ml) (Pharmacia & Upjohn) for 2 days. mDCs were harvested on day 7. Cells were matured with LPS (2 mg/ml) (Sigma-Aldrich), TNF-$\alpha$ (75 ng/ml) (a gift from G. Adolf, Bender, Vienna, Austria), or preincubated for 10 min at room temperature with blocking Ab (10 g/ml), and seeded on the fibronectin-coated plates (3 g/ml) for 45 min at 37°C in the presence of or preincubated for 10 min at room temperature with blocking Ab (10 g/ml), and seeded on the fibronectin-coated plates (3 $\times$ 10$^5$/well) for 45 min at 37°C in the presence or absence of activating Ab (1 g/ml). Non-adherent cells were removed by washing with 0.5% (w/v) BSA in TSM (150 mM NaCl, 10 mM Tris-HCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$ (pH 8.0)) at 37°C. Adherent cells were lysed in 100 $\mu$L of lysis buffer (50 mM Tris, 0.1% (w/v) SDS), and fluorescence was quantified using a cytoflurometer (Applied Biosystems). Results are expressed as the mean percentage of adhesion of triplicate wells.

Transfection and live imaging of DCs

DCs (3 $\times$ 10$^4$) were electroporated with pCMV-$\beta$-actinGFP on day 5 using Amaxa nucleofection (Amaxa Biosystems). Transfected DCs were seeded on fibronectin-coated Willco glass-bottom dishes (Willco Wells). Cells were imaged at 37°C in RPMI 1640 without phenol red (Invitrogen Life Technologies) supplemented with 10% (v/v) FCS using a Zeiss LSM 510-meta microscope equipped with a type S heated stage CO2 controller and PlanApochromatic 63 1.4 NA oil immersion lens (Zeiss). Podosomes were imaged for 20 min with 20-s intervals without PGE$_2$ and for 20 min in the presence of TNF-$\alpha$ (75 ng/ml) at 30-s intervals. Cells were imaged using Zeiss LSM Image Browser version 32 (Zeiss), and images were processed with Image J (version 1.32) software (National Institutes of Health, http://rsb.info.nih.gov/ij/).

Statistical analysis

An ANOVA test or two-tailed Student’s $t$ test was used for statistical analysis.

Results

Induction of high-speed migration in response to DC maturation stimuli

The ability of DCs to migrate is essential for the efficient induction of T cell responses. In earlier work, we have shown that the capacity of DCs to migrate on fibronectin in vitro correlates well with their migratory behavior in vivo (3). Consistent with these previous findings, the majority of the iDCs were slowly migrating with a mean speed of 0.5 $\pm$ 0.1 $\mu$m/min (Fig. 1A). In contrast, DCs matured with a maturation mixture (MCM, PGE$_2$, TNF-$\alpha$), routinely used in clinical applications (3, 26), were highly migratory, showing random migration at a mean speed of 5 $\pm$ 0.5 $\mu$m/min (Fig. 1B). The effects of maturation on cell migration were noticeable after 16 h of stimulation, whereas a full migratory capacity was obtained after 24 h of stimulation (Fig. 1C).
The activity of α5β1 integrin is down-regulated during DC maturation

DC maturation is associated with a significant loss in adhesion to fibronectin (3). The expression of active β1, as determined by an Ab (12G10) that exclusively recognizes the active conformation of this integrin (24), was almost completely absent on mDCs. At the same time, expression levels of β1 integrins were unaffected during maturation (Fig. 2A). These data suggest that the regulation of β1 integrin activity, rather than expression, controls DC adhesion. More importantly, adhesion of mDCs to fibronectin could be completely restored using a β1-activating Ab (TS2/16), while the adhesion of iDCs could not be further enhanced (Fig. 2B). Ab-induced adhesion was still β1 mediated, because an α5-blocking Ab (SAM-1) effectively interfered with this interaction (Fig. 2B). These data demonstrate that, while the α5β1 integrin expressed on mDCs is still capable of mediating adhesion to fibronectin, its activity is specifically down-regulated during DC maturation.

iDCs display podosomes enriched in active β1 integrin

The dramatic changes in cell adhesion and migration, as they occur during DC maturation, are also reflected in the morphology and cytoskeletal organization of these cells. When plated on fibronectin, iDCs exhibited extensive cell spreading accompanied by the formation of numerous podosomes (Fig. 3A, upper panel). In sharp contrast, mDCs were much less spread and no longer organized into podosomes (Fig. 3A, lower panel). Importantly, both the β1 and α5 chain of the fibronectin-binding α5β1 integrin were present in podosomes, and the active form of the β1 integrin, as detected with the 12G10 Ab, was highly enriched in podosomes (Fig. 3B). These results indicate that adhesion of iDCs to fibronectin is mediated by active α5β1 integrin molecules present in these podosomes.

DC maturation signals induce rapid disassembly of podosomes

Because DC maturation leads to dramatic changes in adhesive behavior and migration, we explored the effect of maturation-inducing factors on the actin cytoskeleton. In the immature state, most DCs displayed podosomes. However, in most cells, podosomes were lost within 5–10 min in response to either the maturation mixture or PGE2 alone, while stimulation with TNF-α induced podosome dissolution in a subset of the DCs (Fig. 4, A and B). Prolonged stimulation did not further affect the number of cells displaying podosomes (Fig. 4B). A moderate effect of MCM on podosome dissolution was observed after prolonged stimulation (16 h). These results identify PGE2 as the most active component in the maturation mixture. A concentration of 10 μg/ml PGE2, which is routinely used in the maturation mixture, was the lowest concentration to induce efficient podosome dissolution (Fig. 5). Surprisingly, LPS, a TLR ligand and potent DC maturation factor, failed to induce podosome dissolution after 5–10 min, while prolonged stimulation resulted in a nearly complete loss of podosomes (Fig. 4B). These findings demonstrate that, while most maturation stimuli tested induce dissolution of podosomes, the extent to which
this occurs as well as timing differ between agonists. To examine the kinetics of podosome dissolution in response to maturation stimuli, we expressed a \( \beta_{1} \)-actinGFP construct in iDCs and performed live cell imaging. We observed a rapid turnover of individual podosomes in iDCs expressing \( \beta_{1} \)-actinGFP (lifetime of individual podosomes was ~10 min), while the total number of podosomes remained relatively constant (Fig. 6 and data not shown). Addition of PGE2 or TNF-\( \alpha \) to iDCs caused a rapid and complete dissolution of podosomes within minutes after stimulation (Fig. 4C and data not shown). Together our results show that podosomes in DCs are highly dynamic and that podosome dissolution is the earliest effect observed in response to DC maturation factors such as PGE2 and TNF-\( \alpha \).

Activation of \( \beta_{1} \) integrins interferes with podosome dissolution and high-speed migration

We have shown that \( \beta_{1} \) integrins are prominently present in podosomes and DC maturation is initiated by a loss of these adhesion sites, as well as a global decrease in \( \beta_{1} \) integrin activity. Therefore, we examined whether and how stimulation of \( \beta_{1} \) integrin activity using a \( \beta_{1} \) integrin-activating Ab (TS2/16) affects cytoskeletal changes and the onset of high-speed migration in response to DC maturation. Stimulation with TS2/16 effectively interfered with PGE2-induced podosome dissolution (Fig. 7, A and B) as well as the induction of rapid migration after prolonged incubation (Fig. 7C). These results demonstrate that loss of \( \alpha_{5}\beta_{1} \) integrin activity during DC maturation is key to the induction of high-speed migration.

LPS-induced podosome dissolution and high-speed migration are dependent on the production of PGs

The disassembly of podosomes in response to LPS occurs with a lag phase of 16 h (Fig. 4B), and therefore cannot be a direct consequence of TLR activation. We investigated whether podosome dissolution induced by LPS involves production of PGE2 by the DCs. Therefore, iDCs were seeded on fibronectin and stimulated with LPS for 16 h, either in the presence or absence of indomethacin. This drug inhibits the production of PGE2 and other PGs by

FIGURE 3. iDCs form podosomes enriched in active \( \alpha_{5}\beta_{1} \) integrin. A, iDCs form podosomes on fibronectin. iDCs and mDCs were plated on fibronectin-coated coverslips and stained with an anti-vinculin Ab (green) and phalloidin-Texas Red (red) to detect F-actin. In the lower right corner of the overlay of iDCs, a higher magnification of part of the image is shown. B, Active \( \beta_{1} \) integrin is enriched in podosomes in iDCs. iDCs seeded on fibronectin-coated coverslips were stained with mAbs against \( \alpha_{5} \) (SAM-1), \( \beta_{1} \) (AIIB2), and active \( \beta_{1} \) integrin (12G10) (green). F-actin was detected using phalloidin-Texas Red (red).
inhibiting cyclooxygenases, enzymes involved in the production of PGs (29). Indeed, an effective inhibition of LPS-induced podosome dissolution was observed in the presence of indomethacin (Fig. 8, A and B). In the absence of LPS, no effect of indomethacin on podosomes was seen. Similarly, we observed that indomethacin affects the migratory capacity of LPS-matured DCs (Fig. 8C). These results identify PGs, such as PGE2, as important mediators of maturation-induced DC migration.

**PGE2 and LPS induce podosome dissolution in myeloid DCs**

To examine whether our findings also apply to DCs generated ex vivo, human myeloid DCs were isolated from peripheral blood by magnetic sorting of CD1c-positive/CD19-negative cells (Fig. 9A). These myeloid DCs, although much smaller than the monocyte-derived DCs, similarly formed podosomes on fibronectin, which rapidly dissolved in response to PGE2 stimulation (Fig. 9B and C). Furthermore, PGE2-induced podosome dissolution was efficiently blocked by stimulation with the β3 integrin-activating Ab TS2/16 (Fig. 9C), while stimulation with TS2/16 without PGE2 had no effect on podosomes.

We also investigated the effect of LPS on podosome dissolution in these myeloid DCs. Similar to monocyte-derived DCs, no immediate effects of LPS on podosome dissolution were observed, whereas a complete loss of podosomes was seen after 16-h stimulation with LPS (Fig. 9D). Again, LPS-induced podosome dissolution was effectively inhibited in the presence of indomethacin (Fig. 9D). These results indicate that both in monocyte-derived DCs as well as myeloid DCs, the effects of LPS on podosome dissolution are dependent on the production of PGs by the DC.

**Discussion**

In this study, we report how maturation-induced podosome dissolution and loss of αβ1 integrin activity allow DCs to switch from a strongly adhesive to a highly migratory phenotype. Although a substantial amount of reports describe the biogenesis of podosomes in different cell types (17, 30), mechanisms responsible for podosome disassembly have remained elusive. Consistent with earlier findings in osteoclasts (31), we show that these structures are very dynamic. Although podosomes are generally considered to be features of migratory cells (30, 32), we find a strong inverse correlation between the presence of podosomes and DC migratory capacity. This apparent discrepancy can be easily explained by the fundamental differences in the way iDCs and mDCs interact with the substrate to produce migration. Although iDCs display very strong membrane protrusive activity, as indicated by the presence of lamellipodia and membrane ruffles, strong interactions with the substrate (i.e., podosomes) only allow low-speed migration. Maturing DCs, however, are characterized by a rapid dissolution of podosomes, resulting in high-speed migration. These results identify PGs, such as PGE2, as important mediators of the migratory capacity. This apparent discrepancy can be easily explained by the presence of podosomes and DC migratory capacity.
PGE₂ can be blocked by activation of α₁β₁ integrin. Furthermore, PGE₂-induced podosome dissolution as well as the partial effect of TNF-α on podosome dissolution, the opposite process could be taking place to fuel late endosomal/lysosomal proteins (p61Hck, CD63, and LYAAT-1) localize to podosomes (51–53). Therefore, it has been put forward that late endosomal vesicles fusing with the plasma membrane contribute to podosome formation (53). During podosome dissolution, the opposite process could be taking place to fuel increased vesicle transport as it occurs during DC maturation.

in myeloid DCs, the LPS-induced podosome dissolution is dependent on PG production by the DCs. These findings indicate that ex vivo DCs behave in the same way as monocyte-derived DCs and emphasize the importance of PGE₂ in regulating DC adhesion and migration.

What could be the function of podosomes in iDCs? Podosomes are found in cells that have the capacity to cross tissue boundaries. The observation that podosomes are sites of active matrix degradation (46, 47) and that matrix metalloproteinases are needed for transendothelial migration of leukocytes (48) supports the idea that podosomes are required for crossing tissue barriers. In osteoclasts, podosomes are involved in bone resorption and remodeling (49), while in macrophages podosomes are considered regulators of cell adhesion and migration (50). In addition to a role during transmigration, podosomes in iDCs may contribute to the movement of iDCs within peripheral tissues, while patrolling for Ags.

Disassembly of podosomes as it occurs in response to maturation-inducing agents may be an integral part of the DC maturation process, and may serve to redepoly actin and actin-regulatory proteins to other processes in the cell that require high actin turnover. Podosome biogenesis has been linked to lysosome exocytosis, and late endosomal/lysosomal proteins (p61Hck, CD63, and LYAAT-1) localize to podosomes (51–53). Therefore, it has been put forward that late endosomal vesicles fusing with the plasma membrane contribute to podosome formation (53). During podosome dissolution, the opposite process could be taking place to fuel increased vesicle transport as it occurs during DC maturation.
Two receptors for PGE2 (EP2/4) are expressed on DCs affecting assembly/disassembly of focal adhesions and podosomes (30, 55). Of actomyosin contractility was shown to be important for the as-

than changes in gene expression. Spatial and temporal regulation of DC migration (38, 39, 56). Both of these receptors couple to G protein-coupled receptors and are expressed on myeloid DCs (CD14+/CD1c−/CD11c+). We thank M. Humphries for providing the 12G10 Ab. We thank F. de Lange from the Microscopic Imaging Center of the Nijmegen Centre for Molecular Life Sciences and B. Joosten for assistance with microscopic imaging. We thank I. de Vries, N. Meeuwsen-Scharenborg, A. de Boer, M. Brouwer, and M. van de Rakt for providing DCs.

Disclosures
The authors have no financial conflict of interest.

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