The Journal of Immunology

RESEARCH ARTICLE | AUGUST 01 2006

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J Immunol (2006) 177 (3): 1567–1574.
https://doi.org/10.4049/jimmunol.177.3.1567

A Critical Role for Prostaglandin E_2 in Podosome Dissolution and Induction of High-Speed Migration during Dendritic Cell Maturation[1]

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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 β_1 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 PGE_2, whereas the TLR agonist LPS induces podosome dissolution 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 β_1 integrins. Together, these results show that maturation-induced podosome dissolution and loss of $\alpha_5\beta_1$ 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.**

Dendritic cells (DCs)[5] are the most potent APCs of the immune system (1). Upon Ag uptake and exposure to inflammatory stimuli, these cells undergo a remarkable transition from a tissue-resident, endocytic cell type to a highly migratory APC type, a process known as DC maturation (1, 2). This phenotypical conversion not only involves up-regulation of MHC and costimulatory molecules, but is also accompanied by extensive changes in cytoskeletal organization and cell adhesion (3–6).

The ability of DCs to migrate is essential for the induction of immune responses. Upon Ag recognition and exposure to inflammatory signals, DCs migrate out of the tissues, via the blood or lymph vessels into the lymph nodes, where they activate T cells. We recently demonstrated both in preclinical mouse models (7) and in human cancer patients (3) that DC vaccine efficacy directly correlates with DC maturation and the capacity of these cells to migrate into the lymph nodes. The migration of mature DCs (mDCs) can be defined as high-speed migration (8), comparable to the migration of T cells (9). This type of migration is characterized by short-lived and low-affinity interactions with the substrate. Immature DCs (iDCs), in contrast, show an adhesive and low-speed migratory behavior that is dependent on strong interactions with the extracellular matrix (ECM), similar to fibroblasts (8). β_1 integrins are the main adhesion molecules responsible for these DC-ECM interactions (10, 11). Although the fibronectin-binding integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are both expressed by DCs, we and others have shown that adhesion of DCs to fibronectin is predominantly $\alpha_5\beta_1$ mediated (3, 12, 13). In their high-affinity state, integrins form multimeric complexes in the plasma membrane that enhance binding to the ECM. The integrin cytoplasmic domains in turn recruit a large number of structural and signaling proteins into higher order complexes that connect integrins to the actomyosin cytoskeleton. In most cells, such adhesive contacts are known as focal adhesions (14). However, cells of myeloid origin, such as macrophages, osteoclasts, and DCs, form adhesion complexes distinct from focal adhesions, known as podosomes (15). Unlike focal adhesions, podosomes are not linked to contractile actin stress fibers, but rather consist of an actin-dense core surrounded by a ring of cytoskeletal proteins (16, 17).

Although a large variety of stimuli, including cytokines, CD40 ligation, or TLR ligands, induce DC maturation (18–20), these stimuli not always bring out all the characteristics that mDCs need to fulfill their function (21). Particularly, migration appears to be insufficiently developed in response to some stimuli. To effectively manipulate DC migration in clinical applications, a more detailed knowledge of how maturation factors mobilize DCs is needed. In this study, we investigated the effects of different maturation stimuli on cell adhesion and migration in DCs. We find that high-speed DC migration, induced by maturation signals, requires dissolution of podosomes as well as loss of β_1 integrin activity. In the presence of a β_1-activating Ab, both podosome dissolution and cell migration are effectively inhibited. Moreover, we demonstrate that PGs

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Received for publication January 5, 2006. Accepted for publication April 21, 2006.

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[1] This study was supported by research funding from the Foundation for Fundamental Research on Matter (FOM01FB06-01) and the National Research Council (NWO901-10-092).

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[5] 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.

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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-β_1 integrin-activating epitope (TS2/16) (23), anti-β_1 integrin-blocking epitope (AIIB2) (Developmental Studies Hybridoma Bank), anti-β_1 integrin recognizing an active conformation (12G10) (24) (a gift from M. Humphries, Faculty of Life Sciences, University of Manchester, Manchester, U.K.), anti-α_5 integrin (SAM-1), anti-vinculin (Sigma-Aldrich), mIgG1 (BD Biosciences), mIgG2a, mIgG2b, and rIgG1 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). Monocytes were derived from buffy coats or from a leukapheresis product. Plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker) 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), IL-4 (500 U/ml), and GM-CSF (800 U/ml) (18). iDCs were harvested on day 7. DCs were matured with LPS (2 μg/ml) (Sigma-Aldrich), rTNF-α (75 ng/ml) (a gift from G. Adolf, Bender, Vienna, Austria), or a combination of monocyte-conditioned medium (MCM) (50% v/v), rTNF-α (10 ng/ml), and PGE$_2$ (10 μg/ml) (Pharmacia & Upjohn) for 2 days. mDCs were harvested on day 9. Expression of MHC class I/II, costimulatory molecules, and DC-specific markers on DCs was measured by flow cytometry (data not shown), and the expression of MHC molecules, costimulatory molecules, and DC markers was similar to what was described before (3). iDCs expressed MHC class I and II, the costimulatory molecule CD86, the DC-specific marker CD209/DC-SIGN, and low levels of the costimulatory molecule CD80, and lacked expression of the maturation marker CD83. Maturation resulted in the induction of CD83; up-regulation of MHC class I and II, CD86, and CD80; and a down-modulation of CD209/DC-SIGN expression.

Isolation of myeloid DCs

Myeloid DCs (CD19$^-$CD1c$^+$) were isolated from PBMCs derived from buffy coats. PBMCs were labeled with a FITC-conjugated mAb to CD3 (DakoCytomation), and T and B cells were depleted using anti-FITC and anti-CD19 beads (both Miltenyi Biotec). Myeloid DCs were then isolated from the CD3$^-$CD19$^-$ population using a biotinylated anti-CD1c (BDCA-1) Ab and anti-biotin beads (both Miltenyi Biotec), following the manufacturer's instructions. To assess purity, isolated cells were labeled with streptavidin Cy5 (Jackson ImmunoResearch Laboratories) and a PE-conjugated anti-CD19 Ab (Miltenyi Biotec), PE-conjugated anti-CD14 (Beckman Coulter), or anti-CD11c (BD Biosciences) Abs. Myeloid DCs were identified as cells that expressed CD1c and CD11c, but were negative for CD19. The myeloid DC population thus obtained was 99% pure and represented 0.3% of the total number of PBMCs.

Flow cytometry

Cells (1×10^5) were incubated with 2% (v/v) human serum in PBA (PBS containing 1% (w/v) BSA and 0.05% (w/v) NaN_3) for 15 min at 4°C. After washing with cold PBA, the cells were incubated with primary Ab (5 μg/ml) for 30 min at 4°C. Subsequently, the cells were incubated with FITC-labeled goat anti-mouse. Cells were washed and resuspended in 100 μl of PBA. Fluorescence was measured using a FACSCalibur with CellQuest software (BD Biosciences).

Fluorescence microscopy

Coverslips were coated with fibronectin (20 μg/ml) (Roche) in PBS for 1 h at 37°C. Cells were seeded on fibronectin-coated coverslips, left to adhere for 1–12 h, and fixed in 3.7% (w/v) formaldehyde in PBS for 10 min. Cells were permeabilized in 0.1% (v/v) Triton X-100 in PBS for 5 min and blocked with 2% (w/v) BSA in PBS. The cells were incubated with primary Ab for 1 h. Subsequently, the cells were incubated with Alexa Fluor 488-labeled secondary Abs for 45 min. Subsequently, cells were incubated with Texas Red-conjugated phalloidin for 30 min. Images were collected on a Leica DMRA fluorescence microscope with a ×63 PL APO 1.3 NA oil immersion lens (or a ×40 PL FLUOTAR 1.0 NA oil immersion lens for overview images) and a Cohu high performance integrating charge-coupled device camera (Cohu). Pictures were analyzed with Leica Qfluoro version V1.2.0 (Leica Microsystems) and Adobe Photoshop 7.0 (Adobe Systems) software.

Migration assay

Ninety-six-well flat-bottom plates (Costar) were coated with fibronectin, washed, and blocked with 0.01% (w/v) gelatin (Sigma-Aldrich) in PBS for 30 min at 37°C. Per well, 3×10^3 cells were added and mineral oil was pipetted on top of the medium to prevent pH changes and evaporation of the medium. The cells were monitored with a previously described migration system (28). Cells were recorded for 1 h, followed by analysis of individual cells. The speed was defined as traversed path during the entire experiment divided by the imaging time.

Adhesion assay

Ninety-six-well flat-bottom plates (Costar) were coated with fibronectin and blocked with gelatin. Cells (7×10^6/ml) were labeled with calcein-AM (25 μg/ml) in PBS (Molecular Probes) for 30 min at 37°C, either untreated or preincubated for 10 min at room temperature with blocking Ab (10 μg/ml), and seeded on the fibronectin-coated plates (3×10^4/well) for 45 min at 37°C in the presence or absence of activating Ab (1 μg/ml). Nonadherent 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 μl of lysis buffer (50 mM Tris, 0.1% (w/v) SDS), and fluorescence was quantified using a cytofluometer (Applied Biosystems). Results are expressed as the mean percentage of adhesion of triplicate wells.

Transfection and live imaging of DCs

DCs (3×10^6) were electroporated with pCMV-β-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 CO_2 controller and PlanApochromatic ×63 1.4 NA oil immersion DIC lens (Zeiss). Podosomes were imaged for 20 min with 20-s intervals without PGE$_2$ and for 17 min with 10-s intervals with PGE$_2$ (10 μg/ml). In addition, podosomes were imaged for 60 min without TNF-α, and 20 min in the presence of TNF-α (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 1.32j 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 ± 0.1 μm/min (Fig. 1*A*). In contrast, DCs matured with a maturation mixture (MCM, PGE$_2$, TNF-α), routinely used in clinical applications (3, 26), were highly migratory, showing random migration at a mean speed of 5 ± 0.5 μm/min (Fig. 1*B*). 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. 1*C*).

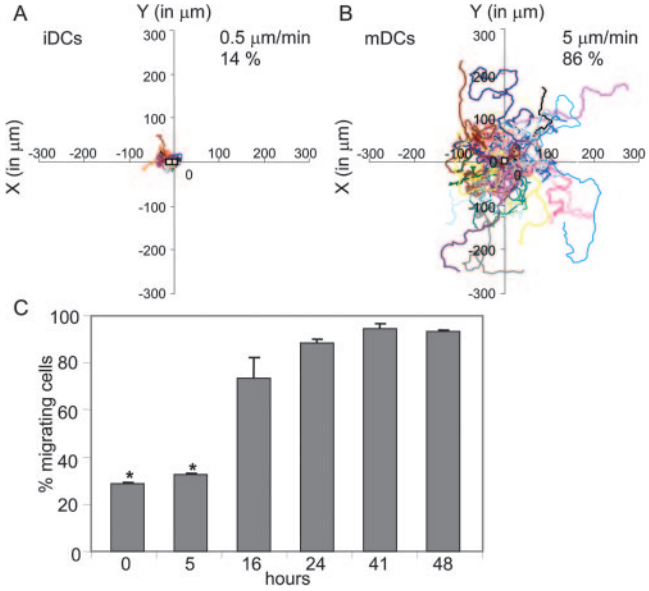

FIGURE 1. mDCs migrate on fibronectin, and migration is initiated after 16–24 h. *A* and *B*, iDCs and mDCs were added to fibronectin-coated plates and monitored for 60 min. Plots of 50 individual tracks, aligned at their starting positions, are shown. The mean speed ± SEM (μm/min) is depicted in the *upper right corner* as well as the percentage of migrating cells ± SD. *C*, DCs were incubated for different time periods (0, 5, 16, 24, 41, and 48 h) with a combination of MCM, PGE_2, and TNF-α, added to fibronectin-coated plates, and migration was monitored for 60 min. The percentage of migrating cells ± SD is depicted. Significant differences ($p < 0.05$) compared with the percentage of migrating DCs after 48 h of maturation are indicated by ∗.

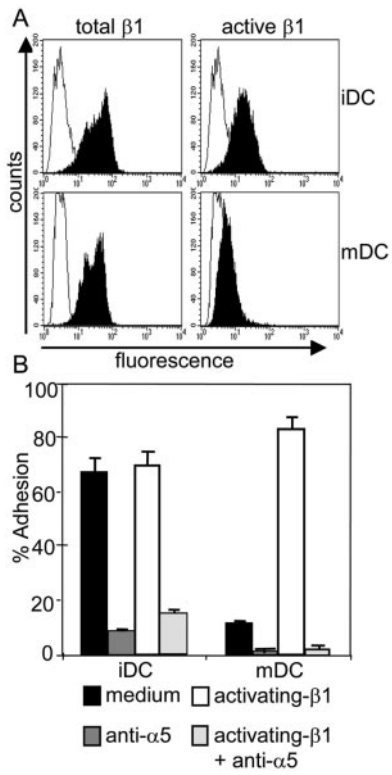

FIGURE 2. Active β_1 integrin is down-regulated upon maturation. *A*, Active β_1 integrin expression on DCs decreases upon maturation. iDCs and mDCs were stained using mAbs recognizing β_1 integrin (AIIB2) or a specific activation epitope of the β_1 integrin (12G10). Expression was determined by flow cytometry. Open graphs represent isotype controls. *B*, Adhesion of mDCs to fibronectin can be enhanced by Ab-induced activation of β_1 integrins. Calcein-AM-labeled iDCs and mDCs were added with or without preincubation with a blocking Ab against α_5 integrin (SAM-1) to fibronectin-coated plates, and incubated in the presence or absence of an anti-β_1 integrin-activating Ab (TS2/16). Cells were lysed and the fluorescence was quantified using a cytofluometer. Results are depicted as the mean percentage of adhesion of triplicate wells ± SD. Adhesion of iDCs and mDCs incubated with SAM-1 before and after activation with TS2/16 is significantly decreased in comparison with nontreated control cells. Adhesion of mDCs using TS2/16 is significantly increased in comparison with adhesion of mDCs without a β_1 integrin-activating Ab ($p < 0.05$).

The activity of $\alpha_5\beta_1$ integrin is down-regulated during DC maturation

DC maturation is associated with a significant loss in adhesion to fibronectin (3). The expression of active β_1, 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 β_1 integrins were unaffected during maturation (Fig. 2*A*). These data suggest that the regulation of β_1 integrin activity, rather than expression, controls DC adhesion. More importantly, adhesion of mDCs to fibronectin could be completely restored using a β_1-activating Ab (TS2/16), while the adhesion of iDCs could not be further enhanced (Fig. 2*B*). Ab-induced adhesion was still $\alpha_5\beta_1$ mediated, because an α_5-blocking Ab (SAM-1) effectively interfered with this interaction (Fig. 2*B*). These data demonstrate that, while the $\alpha_5\beta_1$ 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 β_1 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. 3*A*, *upper panel*). In sharp contrast, mDCs were much less spread and no longer carried podosomes (Fig. 3*A*, *lower panel*). Importantly, both the β_1 and α_5 chain of the fibronectin-binding $\alpha_5\beta_1$ integrin were present in podosomes, and the active form of the β_1 integrin, as detected with the 12G10 Ab, was highly enriched in podosomes (Fig. 3*B*). These results indicate that adhesion of iDCs to fibronectin is mediated by active $\alpha_5\beta_1$ 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 PGE_2 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. 4*B*). A moderate effect of MCM on podosome dissolution was observed after prolonged stimulation (16 h). These results identify PGE_2 as the most active component in the maturation mixture. A concentration of 10 μg/ml PGE_2, 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. 4*B*). These findings demonstrate that, while most maturation stimuli tested induce dissolution of podosomes, the extent to which

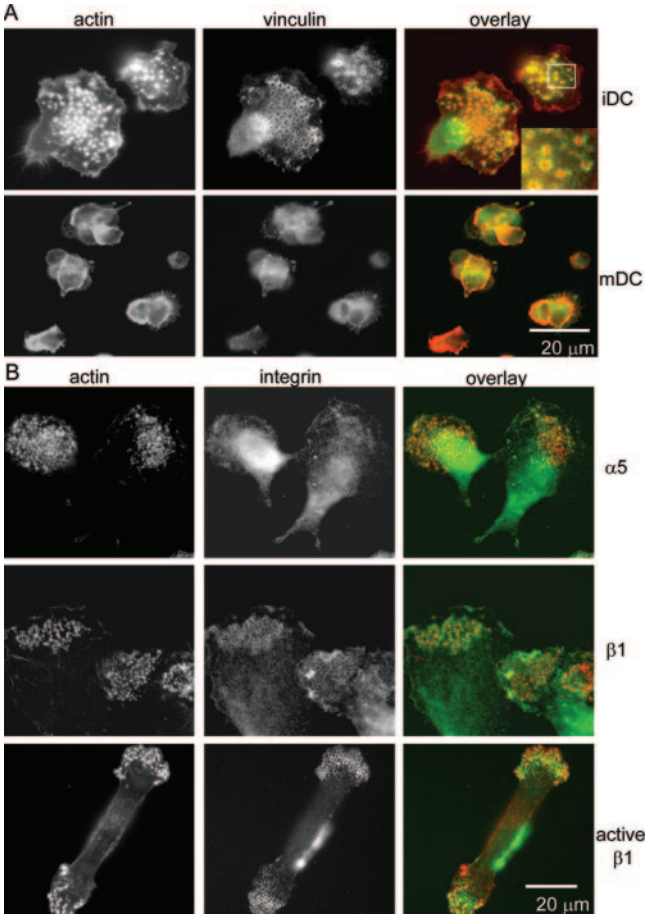

FIGURE 3. iDCs form podosomes enriched in active $\alpha_5\beta_1$ integrin. *A*, iDCs form podosomes on fibronectin. iDCs and mDCs were plated on fibronectin-coated coverslips and stained with an anti-vinculin Ab (green) and phalloidin-Texas Red (red) to detect F-actin. In the *lower right corner* of the overlay of iDCs, a higher magnification of part of the image is shown. *B*, Active β_1 integrin is enriched in podosomes in iDCs. iDCs seeded on fibronectin-coated coverslips were stained with mAbs against α_5 (SAM-1), β_1 (AIIB2), and active β_1 integrin (12G10) (green). F-actin was detected using phalloidin-Texas Red (red).

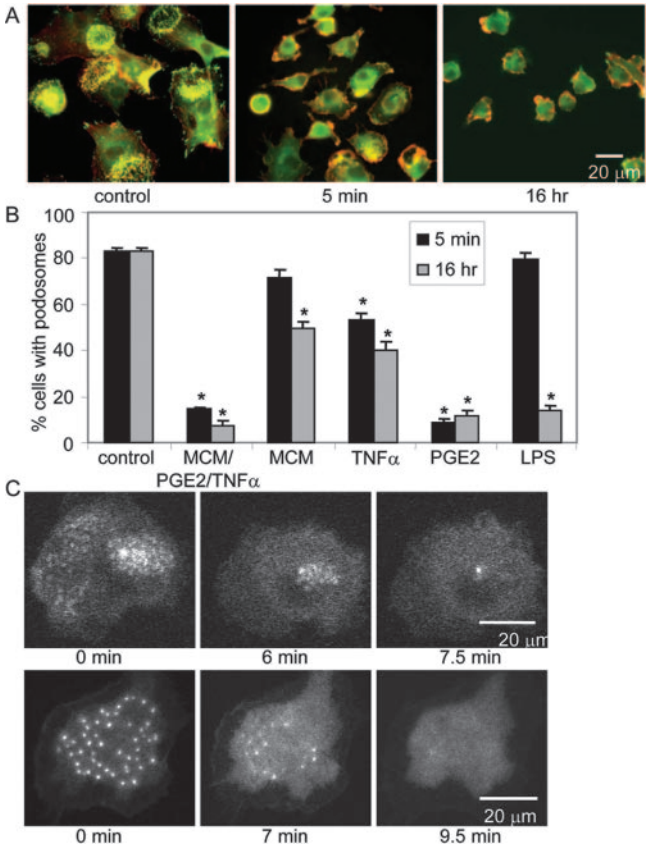

FIGURE 4. Rapid dissolution of podosomes in DCs in response to maturation stimuli. iDCs were seeded on fibronectin-coated coverslips and stimulated for different periods with maturation stimuli. Subsequently, the cells were stained with an anti-vinculin Ab (green) and phalloidin-Texas Red to stain F-actin (red). *A*, iDCs were left untreated or stimulated for 5 min or 16 h with a combination of MCM, PGE$_2$, and TNF-α. Representative images are depicted. *B*, iDCs were either left untreated or stimulated for 5 min or 16 h with a combination of MCM, PGE$_2$, and TNF-α; MCM alone; TNF-α alone; PGE$_2$ alone; or LPS. The number of cells containing podosomes was counted in seven images per condition per experiment. The percentage of cells expressing podosomes $\pm$ SEM is depicted. An average of three experiments is shown. Conditions that are significantly different from the control situation ($p < 0.05$) are indicated with $*$. *C*, iDCs were transfected with β-actinGFP, seeded on fibronectin, and imaged for 60 min before and 20 min after the addition of TNF-α with a 30-s interval or for 20 min with a 20-s interval without PGE$_2$ and 17 min with a 10-s interval with PGE$_2$. Fluorescent images were taken at different time points after TNF-α addition (*upper panel*) and after PGE$_2$ addition (*lower panel*).

this occurs as well as timing differ between agonists. To examine the kinetics of podosome dissolution in response to maturation stimuli, we expressed an β-actinGFP construct in iDCs and performed live cell imaging. We observed a rapid turnover of individual podosomes in iDCs expressing β-actinGFP (lifetime of individual podosomes was $<$10 min), while the total number of podosomes remained relatively constant (Fig. 6 and data not shown). Addition of PGE$_2$ or TNF-α to iDCs caused a rapid and complete dissolution of podosomes within minutes after stimulation (Fig. 4*C* 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 PGE$_2$ and TNF-α.

Activation of β_1 integrins interferes with podosome dissolution and high-speed migration

We have shown that β_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 β_1 integrin activity. Therefore, we examined whether and how stimulation of β_1 integrin activity using a β_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 PGE$_2$-induced podosome dissolution (Fig. 7, *A* and *B*) as well as the induction of rapid migration after prolonged incubation (Fig. 7*C*). 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. 4*B*), and therefore cannot be a direct consequence of TLR activation. We investigated whether podosome dissolution induced by LPS involves production of PGE$_2$ 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 PGE$_2$ and other PGs by

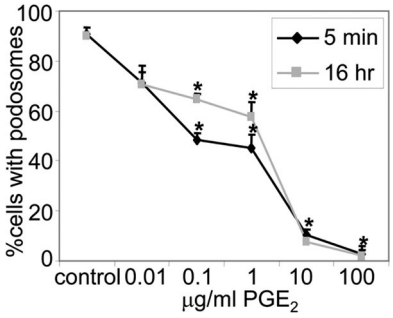

FIGURE 5. PGE_2 is able to mediate complete podosome dissolution at a concentration of 10 μg/ml. iDCs were seeded on fibronectin-coated coverslips and left untreated (control) or stimulated for 5 min or 16 h with different concentrations of PGE_2. Subsequently, the cells were stained with an anti-vinculin Ab and phalloidin-Texas Red to stain F-actin. The number of cells containing podosomes was counted in seven images per condition. The percentage of cells expressing podosomes ± SEM is depicted. One representative experiment is shown. Conditions that are different from the control situation ($p < 0.05$) are indicated with *.

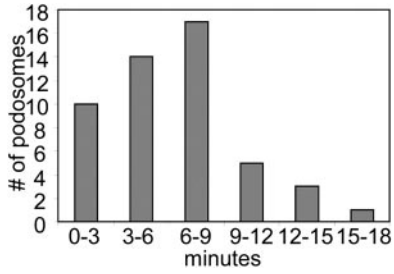

FIGURE 6. The lifetime of individual podosomes is <10 min. iDCs were transfected with β-actinGFP, seeded on fibronectin, and imaged for 60 min with a 30-s interval. The lifetime of individual podosomes in unstimulated cells was determined by counting in how many frames individual podosomes are observed. The lifetime of 50 podosomes was determined, and the distribution of podosome lifetimes is depicted.

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. 8*C*). These results identify PGs, such as PGE_2, as important mediators of maturation-induced DC migration.

PGE_2 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. 9*A*). These myeloid DCs, although much smaller than the monocyte-derived DCs, similarly formed podosomes on fibronectin, which rapidly dissolved in response to PGE_2 stimulation (Fig. 9, *B* and *C*). Furthermore, PGE_2-induced podosome dissolution was efficiently blocked by stimulation with the β_1 integrin-activating Ab TS2/16 (Fig. 9*C*), while stimulation with TS2/16 without PGE_2 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. 9*D*). Again, LPS-induced podosome dissolution was effectively inhibited in the presence of indomethacin (Fig. 9*D*). 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 $\alpha_5\beta_1$ 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 (0.5 μm/min). mDCs display short-lived, weak interactions with the substrate, resulting in high-speed migration, similar to the migration of T cells (9). Consistent with the notion that podosomes restrict high-speed migration, we observed that the active form of the $\alpha_5\beta_1$ integrin is enriched in these structures. Moreover, DC maturation is accompanied by a nearly complete loss of $\alpha_5\beta_1$ integrin-mediated adhesion as well as dissolution of podosomes. As a consequence, mDCs show migration speeds that are 10-fold higher (5 μm/min) than those observed in iDCs. We also show that loss of fibronectin binding, as it occurs during DC maturation, is due to loss of $\alpha_5\beta_1$ integrin activity rather than expression. Furthermore, activation of the $\alpha_5\beta_1$ integrin, using an activating Ab, prevents podosome disassembly and restricts high-speed migration in mDCs. Although preinbucation with this integrin-activating Ab effectively inhibits podosome disassembly, the Ab failed to induce new podosomes in fully mDCs (data not shown). This suggests that, although down-regulation of integrin activity during DC maturation is important for the induction of high-speed migration, $\alpha_5\beta_1$ integrin activation cannot fully reverse the effects of DC maturation on the cytoskeleton. Although there are many examples in which integrin activation of leukocytes triggers the induction of cell migration (33–35), we show in this study that down-regulation of integrin activity may be equally important. Similarly, the importance of integrin deactivation in the induction of cell migration was recently demonstrated for $\alpha_L\beta_2$ integrin (LFA-1). Mice expressing a constitutively active variant of LFA-1 showed defective cell migration and compromised immune function due to impaired deadhesion from its ligand ICAM-1 (36).

A maturation mixture, consisting of MCM, PGE_2, and TNF-α, was used to induce DC maturation. This combination effectively induces all aspects of DC maturation and is used in clinical DC-vaccination studies (26, 37). In this study, we show that PGE_2 is an essential component in the induction of full migratory capacity by this cytokine mixture. DCs matured in the presence of PGE_2 not only migrate more efficiently than without, we also observed that PGE_2 alone is sufficient to induce DC migration. The importance of PGE_2-mediated signaling in DC migration and consequently the induction of a proper immune response is also supported by mouse models. Mice deficient in EP4, a PGE_2 receptor, show impaired skin immune responses due to impaired migration and maturation of Langerhans cells (38). Moreover, it was shown in human DCs that PGE_2 enhances both the expression and the sensitivity of

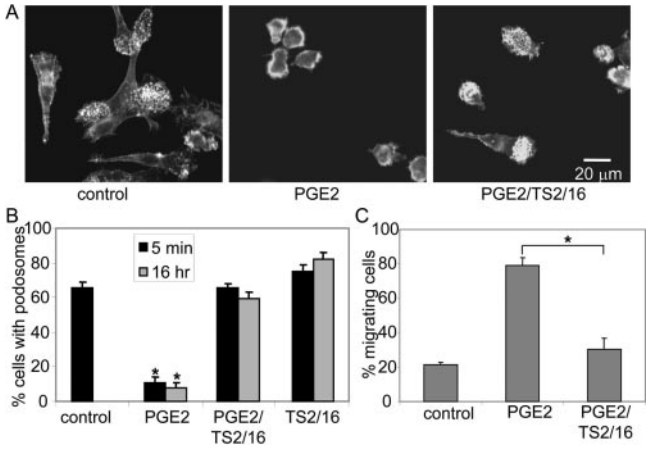

FIGURE 7. Podosome dissolution and migration can be inhibited by activating $\alpha_5\beta_1$ integrin. *A* and *B*, Podosome dissolution in response to PGE_2 can be blocked by activation of $\alpha_5\beta_1$ integrin. iDCs (control) were seeded on fibronectin-coated coverslips and left untreated or stimulated 5 min or 16 h with PGE_2, PGE_2 with the β_1 integrin-activating Ab (TS2/16), or TS2/16 alone. Subsequently, the cells were stained with phalloidin-Texas Red to stain F-actin. The anti-vinculin Ab could not be used because it has the same isotype as the TS2/16 Ab used to activate β_1 integrins. *A*, Representative images of unstimulated DCs or cells stimulated for 16 h with 10 μg/ml PGE_2 in the presence or absence of TS2/16 Ab are shown. *B*, The number of cells containing podosomes was counted in seven images per condition. The percentage of cells expressing podosomes ± SEM is depicted. A representative experiment is shown. Conditions that are significantly different from the control situation ($p < 0.05$) are indicated with ∗. *C*, PGE_2-induced migration is inhibited by activating $\alpha_5\beta_1$ integrin. iDCs (control) or DCs matured with PGE_2 with or without TS2/16 were added to fibronectin-coated plates, and migration was monitored for 60 min. A representative experiment is shown. The percentage of migrating cells ± SD is depicted. Significant differences ($p < 0.05$) are indicated with ∗.

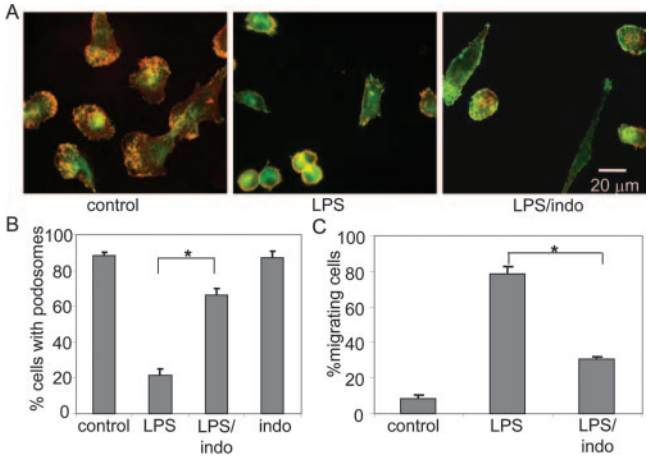

FIGURE 8. LPS-induced podosome disassembly and migration are mediated by PGs. *A* and *B*, LPS-mediated podosome dissolution is PG mediated. iDCs were seeded on fibronectin-coated coverslips and left untreated or stimulated for 16 h with LPS in the presence or absence of indomethacin. Subsequently, cells were stained with an anti-vinculin Ab (green) and phalloidin-Texas Red to stain F-actin (red). *A*, Representative images of DCs either unstimulated (control) or stimulated for 16 h with 2 μg/ml LPS in the presence or absence of 50 μM indomethacin. *B*, iDCs were left unstimulated (control) or stimulated with 2 μg/ml LPS with or without indomethacin (10 μM) or with only indomethacin. The number of cells containing podosomes was counted in seven images per condition per experiment. The percentage of cells expressing podosomes ± SEM is depicted. Significant differences ($p < 0.05$) are indicated by ∗. An average of three experiments is shown. *C*, Migration of LPS-matured DCs is blocked by indomethacin. DCs were matured with 2 μg/ml LPS with or without indomethacin (50 μM). During maturation, indomethacin was refreshed every 12 h. iDCs (control) and mDCs were added to fibronectin-coated plates, and migration with or without indomethacin was monitored for 60 min. The percentage of migrating cells ± SD is depicted. Significant differences ($p < 0.05$) are indicated by ∗.

CCR7 (39–41), the receptor for the chemokines CCL19 and CCL21 that controls DC migration into lymphatic vessels and lymph nodes (42, 43). Consistent with these findings, preliminary results in two melanoma patients participating in a DC-vaccination trail suggest that the percentage of DCs that migrate to the lymph nodes is higher when DCs are matured in the presence of PGE_2 (data not shown). These findings emphasize the importance of PGE_2 in promoting efficient DC maturation and migration, a prerequisite for the induction of an efficient immune response in vivo.

The effect of PGE_2 on podosome dissolution is immediate, as well as the partial effect of TNF-α. This partial response may be due to a lack of TNF-α receptor expression in part of the DCs. In contrast, the effects of LPS are only observed after a long lag time. Importantly, LPS-induced podosome disassembly as well as the induction of high-speed migration can be efficiently inhibited by blocking the production of PGs by the DCs. Furthermore, in response to LPS stimulation, DCs start to produce PGE_2 after 6 h and this production is maximal after 24 h (44, 45), which coincides with the loss of podosomes and the induction of migration. These results demonstrate that pathogen-derived molecules, such as LPS, induce podosome dissolution and high-speed migration by stimulating the production of PGE_2 by DCs during an inflammatory response.

Ex vivo generated myeloid DCs form podosomes on fibronectin similar to monocyte-derived DCs and rapidly dissolve their podosomes in response to PGE_2. Furthermore, PGE_2-induced podosome dissolution can be inhibited by activating β_1 integrins, indicating that also in myeloid DCs regulation of the activity of β_1 integrins is important for adhesion and migration. Moreover, also 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 PGE_2 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 redeploy 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.

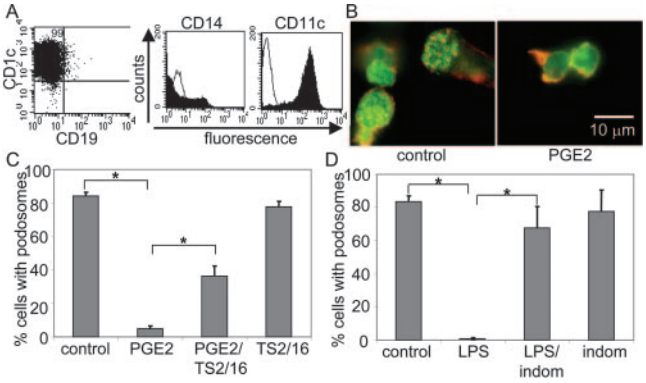

FIGURE 9. Podosome dissolution in myeloid DCs. *A*, Myeloid DCs (CD1c/BDCA-1-positive cells) were isolated from PBMCs and analyzed by flow cytometry. These myeloid DCs were 99% pure ($CD19^-CD1c^+$) and expressed high levels of CD11c, while a subset expressed CD14. *B*, Myeloid DCs were seeded on fibronectin-coated coverslips and stimulated with PGE_2 for 5 min. Subsequently, cells were stained with an anti-vinculin Ab (green) and phalloidin-Texas Red to detect F-actin (red). Representative images are shown. *C*, Myeloid DCs were seeded on fibronectin-coated coverslips and stimulated with PGE_2 for 5 min in the presence or absence of TS2/16 Ab. Subsequently, the cells were stained with phalloidin-Texas Red to detect F-actin (red). The number of cells containing podosomes was counted in 15 images per condition. The percentage of cells expressing podosomes ± SEM is depicted. Conditions that are significantly different ($p < 0.05$) are indicated with ∗. *D*, Myeloid DCs were seeded on fibronectin-coated coverslips and stimulated with LPS in the presence or absence of indomethacin. Subsequently, cells were stained with an anti-vinculin Ab (green) and phalloidin-Texas Red to detect F-actin (red). The number of cells containing podosomes was counted in seven images per condition. The percentage of cells expressing podosomes ± SEM is depicted. Conditions that are significantly different ($p < 0.05$) are indicated with ∗. One representative experiment is shown.

Whereas the effects of LPS stimulation on podosome disassembly in human DCs appear to be indirect and require 16-h incubation, in mouse DCs a rapid, but transient dissolution of podosomes is seen minutes after LPS stimulation (6). We speculate that these differences in LPS responsiveness between mouse and human DCs reflect variations in LPS-mediated signal transduction between species (54). It will be interesting to determine whether prolonged exposure of mouse DCs to LPS also leads to a sustained podosome loss and whether these late responses are also dependent on the production of PGs such as PGE_2.

How does PGE_2 induce podosome disassembly? The effects of PGE_2 on podosomes occur within minutes, which implies that these are a direct consequence of PGE_2 receptor activation rather than changes in gene expression. Spatial and temporal regulation of actomyosin contractility was shown to be important for the assembly/disassembly of focal adhesions and podosomes (30, 55). Two receptors for PGE_2 (EP2/4) are expressed on DCs affecting DC migration (38, 39, 56). Both of these receptors couple to $G\alpha_s$ to increase cAMP levels, leading to activation of protein kinase A (57). Changes in activity regulate actomyosin contractility, either by affecting the activity of the small GTPase RhoA, a key regulator of myosin II function, or by inactivation of myosin L chain kinase (reviewed by Burridge and colleagues (58) and references therein). Future experiments will therefore be aimed at determining how these signal transduction pathways contribute to DC adhesion and migration.

Acknowledgments

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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