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Mature Dendritic Cells Express Functional Thrombin Receptors Triggering Chemotaxis and CCL18/Pulmonary and Activation-Regulated Chemokine Induction[1]

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Protease-activated receptors (PARs) are a family of G protein-coupled receptors that are activated by serine protease-mediated proteolytic cleavage of their extracellular domain. We have previously characterized the expression and function of PARs in human monocytes and macrophages, yet information about PARs in dendritic cells (DC) is scarce. Monocyte-derived immature DC do not express PARs. Upon maturation with LPS, but not with TNF-α or CD40 ligand, DC express PAR1 and PAR3, but not PAR2 or PAR4. Stimulation of DC with the serine protease thrombin or PAR1-activating peptide elicits actin polymerization and concentration-dependent chemotactic responses in LPS-, but not in TNF-α-matured DC. The thrombin-induced migration is a true chemotaxis with only negligible chemokinesis. Stimulation of PARs with thrombin or the respective receptor-activating peptides activates ERK1/2 and Rho kinase as well as subsequent phosphorylation of the regulatory myosin L chain 2. The ERK1/2- and Rho kinase 1-mediated phosphorylation of myosin L chain 2 was indispensable for the PAR-mediated chemotaxis as shown by pharmacological inhibitors. Additionally, thrombin stimulated the Rho-dependent release of the CC chemokine CCL18/pulmonary and activation-regulated chemokine, which induces chemotaxis of lymphocytes and immature DC as well as fibroblast proliferation. The colocalization of CD83$^+$ DC with CCL18 in human atherosclerotic plaques revealed by immunofluorescence microscopy combined with the presence of functionally active thrombin receptors on mature DC point to a previously unrecognized functional role of thrombin in DC biology. The thrombin-induced stimulation of mature DC may be of particular relevance in atherosclerotic lesions, which harbor all components of this novel mechanism. ***The Journal of Immunology,*** **2008, 181: 1215–1223.**

Thrombin is generated at sites of vascular injury and during inflammation (1, 2). When generated in close proximity to its cellular receptors (3, 4), thrombin triggers not only increased fibrin deposition, but also activation of cells expressing protease-activated receptors (PARs),[4] which include endothelial and vascular smooth muscle cells as well as various blood cells (1, 2, 5).

Binding of thrombin to its respective PARs leads to cleavage of the N-terminal exodomain, unmasking a new N terminus that acts as tethered ligand. Receptor-activating peptides (AP) mimic the newly formed N-terminal sequence of the receptor and activate PARs independent of protease activity and receptor cleavage (1, 2). Among the four members of PARs, PAR1, PAR3, and PAR4 are activated by thrombin, whereas PAR2 is activated, by trypsin, factor VIIa or Xa, as well as mast cell tryptase (1, 2).

Dendritic cells (DC) are essential for the induction of the adaptive immune response (6, 7). On the basis of their phenotype and their ability to prime naive T cells, they are commonly subdivided into immature and mature DC (mDC) (8). Ags, pathogens, LPS, TNF-α, and other factors induce functional changes culminating in the transition from the Ag-capturing immature to the Ag-presenting mDC (7, 8).

Small numbers of DC are localized in the intima of apparently normal, nondiseased arteries (9–11). Interestingly, atherosclerosis-prone regions of the murine vessel wall contain abundant DC, suggesting a role of these cells in the process of atherogenesis (12). In atherosclerotic arteries, the number of DC further increases, indicating that some of them do not migrate to lymph nodes, but remain in the vessel wall, where they could activate T cells directly within the intima (10, 11, 13). DC-T cell interactions were shown to be frequent in rupture-prone plaque regions, suggesting that this could contribute to plaque destabilization (14). LPS and endogenous ligands such as minimally modified low density lipoprotein activate TLR4, whereas genetic deficiency of TLR4 or MyD88 involved in TLR signaling reduces atherosclerosis as well as the plaque areas in apoE$^{-/-}$ mice (15, 16). Further evidence for a link between adaptive immune responses and atherogenesis comes from the enhanced TLR expression in murine and human atherosclerotic lesions. Although pivotal roles for DC and TLR signaling have been claimed in the development of atherosclerosis (13, 17), little is known about the induction of DC stimulation and accumulation.

Immature DC (iDC) express only small amounts of PAR1 and PAR3 mRNA, but no detectable PAR proteins on their surface (5). On the other hand, a subset of DC may express PAR2. Thus, an endogenous serine protease acting on PAR2 seems to stimulate DC development from murine bone marrow progenitor cells (18). So far, little is known about the expression and function of PARs in DC.

In this study, we report expression and function of thrombin receptors in DC matured by different stimuli. We demonstrate for the

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[4] Abbreviations used in this paper: PAR, protease-activated receptor; DC, dendritic cell; iDC, immature DC; mDC, mature DC; ROCK, Rho kinase; MLC2, myosin L chain 2; AP, activating peptide; PARC, pulmonary and activation-regulated chemokine; CD40L, CD40 ligand.

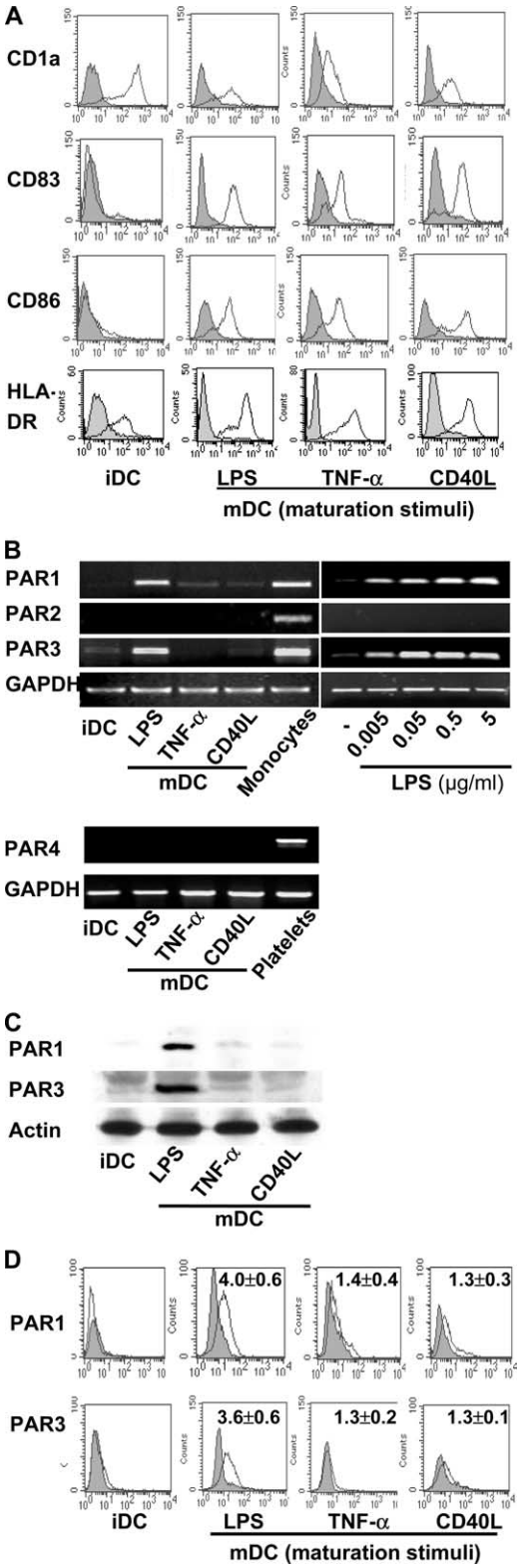

FIGURE 1. Maturation of monocyte-derived iDC with LPS induces PAR1 and PAR3 expression. iDC were differentiated from monocytes using GM-CSF and IL-4. Maturation of iDC was induced either by 0.5 μg/ml LPS, 50 ng/ml TNF-α, or 0.5 μg/ml CD40L for 48 h. Flow cytometry of the DC markers HLA-DR, CD83, and CD86 and CD1a (*A*). Shaded peaks represent isotype controls. RT-PCR analysis of PAR mRNA expression in iDC and mDC by either LPS (0.5 μg/ml), TNF-α, or CD40L (*left panel*) or the indicated concentrations of LPS (*right panel*) for 48 h (*B*). Monocytes served as positive control. RT-PCR analysis did not detect PAR4 expression. PAR4 expression in platelets served as positive control. GAPDH mRNA, normalization. Western blot analysis of PAR1 and PAR3 expression in whole cell lysates (*C*). Actin, loading control. Flow cytometry of PAR1 and PAR3 expression on the DC surface (*D*). Shaded peaks, isotype controls. The inserts represent the mean fluorescence index calculated as ratio of mean fluorescence intensity of PAR1 or PAR3 expression compared with control IgG and presented as mean ± SEM of three to seven experiments.

first time that in LPS-matured DC thrombin triggers chemotactic responses as well as an increased release of the CC chemokine CCL18, also known as pulmonary and activation-regulated chemokine (PARC), for which no murine homolog exists (19). Our data provide insight into novel aspects of DC function linking the coagulation system to the adaptive immune response. The findings might be of particular significance in atherosclerotic lesions, where all necessary components of this new activation mechanism are colocalized. Support for such a mechanism comes from immunofluorescence microscopy of plaque material from human carotid endarterectomies showing colocalization of matured $CD83^+$ DC, thrombin receptors, and CCL18.

Materials and Methods

Differentiation of DC

DC were differentiated from human monocytes obtained from buffy coats with 1000 U/ml GM-CSF (Berlex Laboratories) and 25 ng/ml IL-4 (Pierce) as described previously (5). The differentiation was confirmed by flow cytometric analysis of CD14 (mouse anti-CD14; Dianova), CD1a, HLA-DR, CD83, and CD86 (BD Biosciences). As secondary Abs served PE-conjugated donkey anti-mouse and donkey anti-rabbit $F(ab')_2$ (Dianova). iDC were used after 6 days. Maturation was induced by incubation for an additional 48 h using either 0.5 μg/ml LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich), 50 ng/ml TNF-α (PeproTech), or 0.5 μg/ml CD40 ligand (CD40L; PJK) (20) Cells were kept in FCS-free RPMI 1640 for 6 h before each experiment. We excluded platelet contamination by analysis of CD41 that remained undetectable both by RT-PCR and flow cytometry (data not shown).

mRNA detection

Total RNA was extracted using TRIzol (Invitrogen). Total RNA (1.5 μg) was used and specific primers for PAR1, PAR2, PAR3, PAR4, and GAPDH (ThermoHybaid) for normalization (5). Primers for CCL18 were as described elsewhere (21). To exclude genomic DNA contamination, all RNA samples were pretreated with DNase I (Invitrogen). The identity of the PCR products was confirmed by sequencing (Applied Biosystems Prism 310). Platelets isolated as described served as positive control for PAR4 mRNA amplification (5). For quantitative real-time PCR, reactions were run on a real-time PCR system (Applied Biosystems 7300), detected with SYBR green (Applied Biosystems), and relative gene expression was determined by normalizing to GAPDH using the ΔC_T method.

Protein expression

Protein expression was analyzed by Western blot immunoblotting and flow cytometry with a FACScan (BD Biosciences) (5). For the analysis of phosphorylation of ERK1/2, p38, and myosin L chain (MLC) 2 (Cell Signaling Technology), mDC (0.8×10^6 cells, day 6) were kept without stimuli in FCS-free medium for an additional 6 h before stimulation with thrombin (Enzyme Research Laboratories) or PAR-specific APs. Alternatively, cells were pretreated for 15 min with 10 μM of either Y27632, U0126, or 1 μM peptide 18 (Calbiochem) before treatment. After indicated times, the cells were lysed for Western blot immunoblotting. Actin served as loading control (Chemicon International). Expression of PARs on the cell surface was analyzed by flow cytometry using mouse anti-PAR1 (Beckman Coulter) and rabbit anti-PAR3 (Santa Cruz Biotechnology) (5). For the quantification of CCL18 secretion, supernatants of DC were harvested 24 h after treatment with thrombin (3 U/ml), PAR1-AP (TFLLRNPNDK), or PAR3-AP (TFRGAP; ThermoHybaid), each at 100 μM and stored at −20°C until analyzed by ELISA (R&D Systems). Hirudin (10 U/ml; Calbiochem), anti-PAR1-neutralizing Ab WEDE15 (50 μg/ml; Beckman Coulter), or PAR1-specific inhibitor SCH 79797 (10 μM; Tocris) (22) added 20 min before stimulation were used to ensure the specificity of the effects observed.

Cytosolic calcium levels

Washed cells were loaded with fura-2 acetoxymethyl ester (Molecular Probes), challenged with either thrombin (1–10 U/ml) or the positive control WKYMVm (10 nM; Bachem) (23) and cytosolic Ca^{2+} concentrations were analyzed and calculated as previously described (5).

Actin polymerization

DC (5×10^6 cells/ml) were kept at 37°C for 5 min before the addition of the chemoattractants thrombin (3 U/ml), PAR-1AP (100 μM), PAR-3AP (100 μM), or the positive control WKYMVm (10 nM) (23). Hirudin (10 U/ml) was used as a specific inhibitor of thrombin. At the indicated time points, the reactions were stopped by addition of paraformaldehyde. After washing, the cells were permeabilized in ice-cold PBS/0.1% Triton X-100, stained with fluorescein-labeled phalloidin (Molecular Probes), and analyzed by flow cytometry (FACScan). For each time point, the mean fluorescence intensity was normalized to control, i.e., nonstimulated cells at time point 0. Alternatively, DC (0.75×10^6 cells/chamber) were seeded onto Falcon culture slides (BD Biosciences) on day 6, just before LPS maturation. Two days later, cells were stimulated with thrombin (3 U/ml), fixed, permeabilized, stained with Texas Red phalloidin, counterstained with Hoechst 33258, and analyzed by microscopy using an Axiophot 2 (Zeiss) and AxioVision 4.5.

ROCK assay

DC were stimulated either with thrombin (3 U/ml), PAR1-AP (300 μM), or PAR3-AP (300 μM) for the indicated time. The cells were lysed, divided into two aliquots, and precleared with protein A or G Sepharose beads. Rho kinase (ROCK) 1 and ROCK2 were immunoprecipitated using rabbit anti-ROCK1 (Sigma-Aldrich) or goat anti-ROCK2 (Santa Cruz Biotechnology) and protein A (ROCK1) or G (ROCK2) Sepharose beads (GE Healthcare Bio-Sciences). The kinase activity was analyzed using [γ-^{32}P]ATP and 0.5 μg of recombinant MYPT1 (Upstate Biotechnology) as substrate (24). Phosphorylated substrate was visualized by phosphor imaging. Western blot immunoblotting of ROCK1 and ROCK2 ensured specific immunoprecipitation.

Migration assays

Cell migration was evaluated using tissue culture-treated 24-well Transwell plates (Costar) with polycarbonate membranes (pore size, 5 μm). DC were suspended in RPMI 1640 containing 0.1% BSA at a concentration of 2×10^6 cells/ml, and 100 μl was added to the upper compartment of each well (25). When cells were pretreated with kinase inhibitors, these were added to the upper and lower compartments 15 min before addition of the respective chemoattractant. For checkerboard analysis, increasing concentrations of thrombin were added to the upper and lower compartments. DC were allowed to migrate for 4 h, fixed with paraformaldehyde, stained with hematoxylin (Accustain; Sigma-Aldrich), and migrated cells were counted with a standard microscope (Olympus CH-2; ×1000 magnification, oil immersion) (25).

Fluorescence microscopy

Cryosections (5 μm) were obtained from frozen human carotid endarterectomy specimens. The sections were fixed in acetone, blocked with 5% goat serum, and incubated with Cy5-coupled mouse anti-CD83 IgG1 (551058; BD Biosciences) as a marker for mDC (7, 20) or FITC-coupled mouse anti-CCL18 IgG1 (IC394F; R&D Systems) in PBS/0.3% Triton X-100. Alternatively, anti-PAR1 (WEDE15), anti-PAR3 (H-103; Santa Cruz Biotechnology), or control IgG (Dianova) followed by FITC-conjugated F(ab′)$_2$ (Dianova) were used. Nuclei were counterstained with Hoechst 33258. Fluorescence microscopy was performed with an Axiophot 2. Control sections were stained with the same concentrations of Cy5- and FITC-labeled control IgG (Dianova). Specimens from nine patients were analyzed.

Statistical analysis

Statistical significance was calculated with the Newman-Keuls test. Data shown represent the mean ± SEM where applicable. Differences were considered significant for $p < 0.05$.

Results

LPS-induced maturation triggers PAR1 and PAR3 expression in monocyte-derived DC

Analyzing the expression of PARs in human APCs, we have previously shown that differentiation of monocytes into iDC is accompanied by down-regulation of PAR1 and PAR3 on the cell surface (5). In this study, we investigated the induction of thrombin receptors in DC matured by different stimuli.

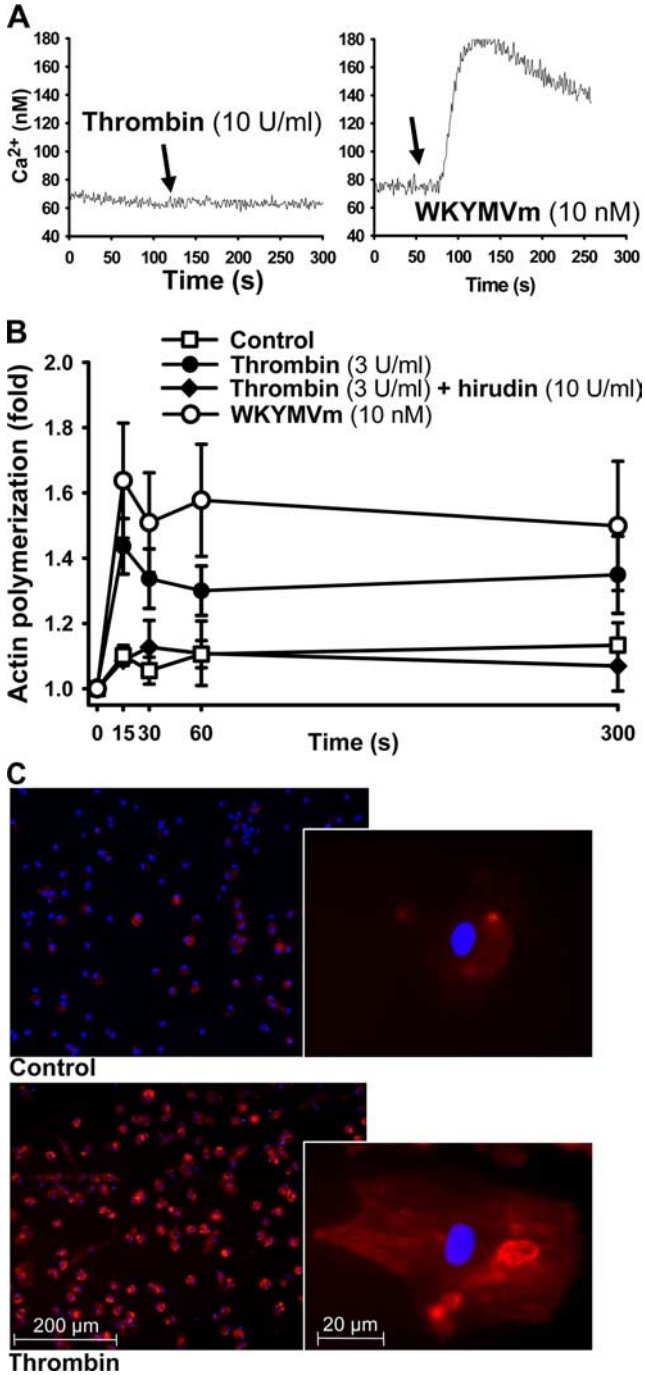

FIGURE 2. Thrombin fails to increase cytosolic Ca^{2+} levels yet induces actin polymerization in LPS-matured DC. DC loaded with fura-2 acetoxymethyl ester were stimulated with thrombin. DC stimulated with the peptide WKYMVm served as positive controls (*A*). One of three independent experiments is shown. Actin polymerization in mDC stimulated with thrombin in the presence or absence of hirudin was analyzed by flow cytometry (*B*). WKYMVm, positive control. The cells were stained with fluorescein-labeled phalloidin. Results are presented as mean fluorescence intensity of four to seven independent experiments ± SEM. Fluorescence microscopy of actin polymerization in LPS-matured DC 1 min after thrombin (3 U/ml) stimulation (*C*). F-actin was visualized with Texas Red-conjugated phalloidin (red). Nuclei were counterstained with Hoechst 33258 (blue). Original magnification, ×200 and ×1000.

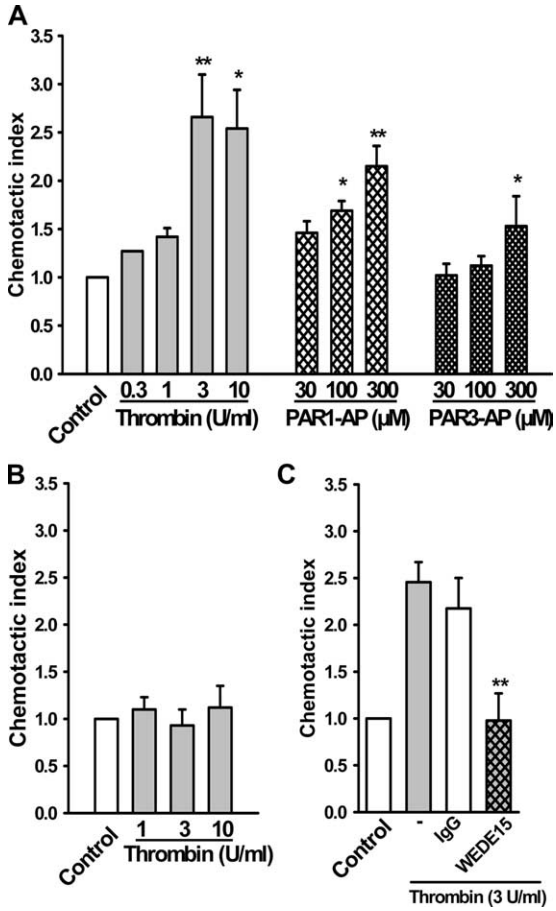

FIGURE 3. Thrombin induces chemotaxis of LPS-, but not of TNF-α-matured DC. Migration of mDC was analyzed in Transwell chambers (pore size, 5 μm). After migration (4 h), the cells were fixed, stained, and those migrated through the membrane were counted. The data are presented as chemotactic index, the ratio between the cells migrated to the chemotactic stimulus and the random migration (control). Thrombin, PAR1-AP, and PAR3-AP induce concentration-dependent chemotaxis of LPS-matured DC (*A*). Thrombin does not induce chemotaxis of TNF-α-matured DC (*B*). Thrombin-induced chemotaxis is inhibited by PAR1-neutralizing Ab WEDE15 (50 μg/ml) (*C*). Results are mean ± SEM of four to six independent experiments. ∗, $p < 0.05$ and ∗∗, $p < 0.01$ vs controls.

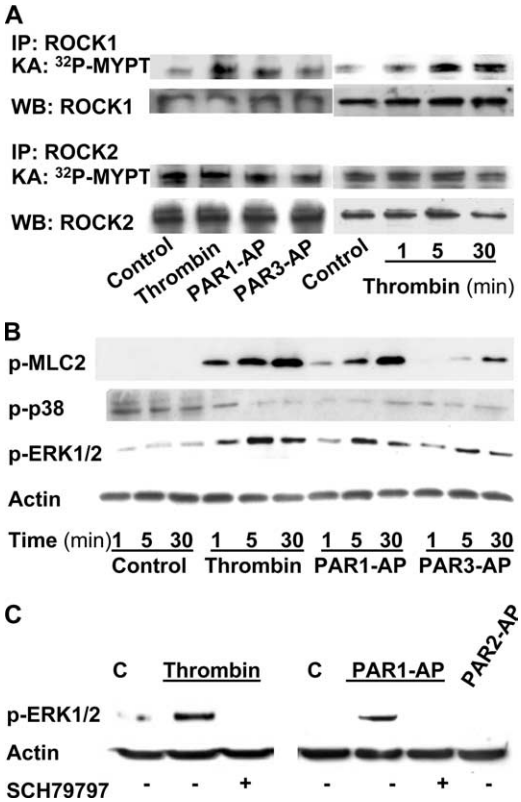

FIGURE 4. Thrombin activates kinases ROCK1 and ERK1/2 and triggers phosphorylation of regulatory MLC. Rho kinase assay (*A*). mDC were stimulated with either thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each at 100 μM) for 20 min or thrombin (3 U/ml) for the indicated time. The cells were lysed and ROCK1 or ROCK2 were immunoprecipitated. Kinase activity was analyzed using MYPT as substrate. Phosphorylated substrate was visualized by phosphor imaging. Western blotting of ROCK1 and ROCK2 ensured specific immunoprecipitation. IP, Immunoprecipitation; KA, kinase assay; WB, Western immunoblot. Western immunoblots of phosphorylated regulatory MLC (MLC2), ERK1/2, and p38 MAPK in mDC-treated either with thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each at 100 μM) (*B*). Actin, loading control. Thrombin (3 U/ml) and PAR1-AP (100 μM)-induced p-ERK1/2 phosphorylation is inhibited by the specific PAR1 antagonist SCH 79797 (10 μM) (*C*). Each experiment is representative of three.

We confirmed maturation of iDC by either LPS, TNF-α, or CD40L by flow cytometry showing up-regulation of the HLA-DR, MHC class II costimulatory molecules CD83 and CD86 (Fig. 1*A*) and down-regulation of CD1a (7, 20). When DC were matured either by exposure to TNF-α or CD40L, RT-PCR did not yield significant amounts of PAR1, PAR2, or PAR3 amplification products (Fig. 1*B*). In contrast, maturation with LPS concentration dependently increased both PAR1 and PAR3 mRNA levels (Fig. 1*B*). The differential induction of PAR mRNA by the various maturation stimuli was reflected by analogous changes at the protein level of matured DC as shown by immunoblots of whole cell lysates (Fig. 1*C*) and by flow cytometric analysis of the PAR1 and PAR3 expression on the cell surface (Fig. 1*D*). At the concentrations used, LPS did not affect the viability of the matured DC as measured by the XTT (formazan) assay and by annexin V binding (data not shown).

Table I. *Checkerboard analysis of thrombin-induced migration of mDC*[a]

Below Membrane	Above Membrane			
	Thrombin (U/ml)			
Thrombin (U/ml)	0	1	3	10
0	1.00	0.99 ± 0.24	1.12 ± 0.18	0.97 ± 0.12
1	1.20 ± 0.25	1.08 ± 0.16	0.93 ± 0.18	1.35 ± 0.36
3	2.52 ± 0.20	2.30 ± 0.44	1.52 ± 0.36	1.34 ± 0.34
10	2.62 ± 0.36	2.43 ± 0.29	2.15 ± 0.47	1.30 ± 0.25

[a] The concentrations of thrombin were added to the upper and/or lower compartment of the Transwell chambers. The chemotactic index compared to nontreated cells is given; mean ± SEM of four independent experiments.

Similar to iDC (5), PAR4 mRNA remained undetectable in mDC, but was easily identified in platelets, which served as positive control (Fig. 1*B*). Thus, similar to monocytes and macrophages, mDC do not express PAR4, yet upon LPS-induced maturation, they do express PAR1 and PAR3.

Thrombin elicits actin polymerization in LPS-matured DC

In various cells including monocytes, signaling via PARs increases cytosolic Ca^{2+} levels (1, 5, 26, 27). However, stimulation of mDC with thrombin (1–10 U/ml) did not elicit any cytosolic Ca^{2+}

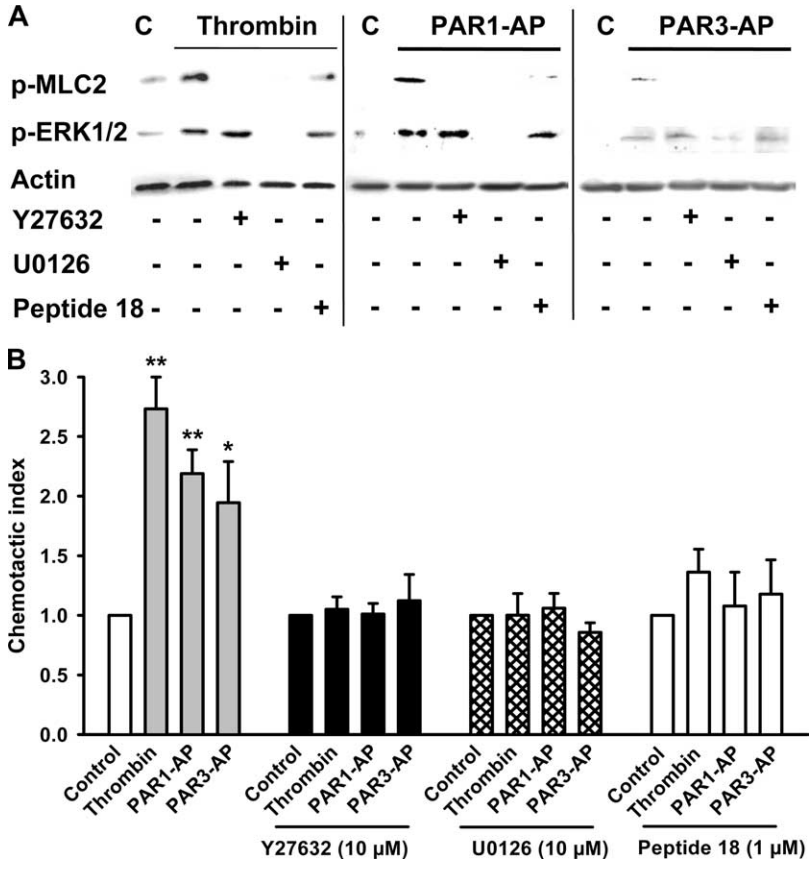

FIGURE 5. Thrombin-induced chemotaxis of LPS-matured DC is critically dependent on ROCK, ERK1/2, and MLCK signaling. Thrombin-induced phosphorylation of MLC2 is inhibited by inhibitors of ROCK, MEK, and MLCK (*A*). Western immunoblot of DC pretreated for 15 min with either ROCK inhibitor Y27632, MEK inhibitor U0126 (each 10 μM), or the MLCK inhibitor peptide 18 (1 μM) and then treated with either thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each at 100 μM) for 20 min. Phosphorylated MLC2 and phosphorylated/activated ERK1/2 were analyzed by immunostaining. Actin, control. One of three independent experiments is shown. Chemotaxis (*B*). Cells were preincubated with inhibitors for 15 min and then allowed to migrate toward thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each at 100 μM). Results are mean ± SEM of three independent experiments.

increase, whereas the same cells clearly responded to the chemotactic peptide WKYMVm (23) as positive control (Fig. 2*A*).

Thrombin-induced chemotaxis is well established in monocytes and vascular smooth muscle cells (1, 2). Cell migration requires actin polymerization at the leading edge of the polarized cells and myosin activation at the rear, thereby enabling cell contraction and detachment (28). Within 15 s of thrombin stimulation, we observed a sustained polymerization of actin, which was completely inhibited by the specific thrombin inhibitor hirudin (Fig. 2*B*). The actin polymerization increased 30 s after stimulation to 1.36 ± 0.04 for thrombin (3 U/ml), 1.25 ± 0.02 for PAR1-AP, and 1.21 ± 0.10 for PAR3-AP (fold increase, both at 100 μM). Similarly, WKYMVm (23) induced an equally strong response (Fig. 2*B*). The thrombin-induced actin polymerization became clearly visible in permeabilized mDC stained with fluorescence-labeled phalloidin (Fig. 2*C*). A similar increase in actin polymerization was observed in monocytes stimulated with thrombin (3 U/ml; data not shown).

Thrombin stimulates migration of LPS-mDC

Thrombin induced a concentration-dependent migration in LPS-matured (Fig. 3*A*), but not in TNF-α-matured DC (Fig. 3*B*), implying that the cell migration depends on the differential PAR expression triggered by the maturation stimuli. The thrombin-induced locomotion was clearly dependent on a positive concentration gradient of thrombin between the lower and upper compartments of the chambers as confirmed by checkerboard analysis (Table I). When equal concentrations of thrombin were added to the upper and lower compartments, higher concentrations of thrombin (3–10 U/ml) initiated only a slightly higher random cell migration. Therefore, only a negligible component of the thrombin-induced DC migration is attributable to chemokinesis. Thus, thrombin induces a true chemotactic response in LPS-matured DC.

To confirm the putative role of PARs in this effect, we also tested a specific PAR1-AP for its chemotactic activity. PAR1-AP (30–300 μM) elicited a concentration-dependent chemotactic response in LPS-matured DC (Fig. 3*A*), and anti-PAR1-neutralizing Ab WEDE15 (5) completely inhibited the thrombin-induced migration (Fig. 3*C*), indicating that thrombin activates DC via PAR1. Only at 300 μM PAR3-AP, which mimics the sequence of the human PAR3-tethered ligand, triggered a modest chemotactic response in the LPS-matured DC (Fig. 3*A*).

Thrombin activates ROCK1, ERK1/2, and triggers phosphorylation of MLC2

Activation of Rho GTPases in migrating cells is essential for the formation of stress fibers, focal adhesion complexes, and contractile actin-myosin filaments (28, 29). Rho exerts its effects through activation of the ROCKs ROCK1 and ROCK2. ROCKs are serine/threonine kinases, which activate the regulatory myosin L chain (MLC2) either by direct phosphorylation or by phosphorylation and inactivation of the myosin-binding subunit (MYPT) of the MLC phosphatase; such inactivation of the MLC phosphatase leads to decreased MLC2 dephosphorylation and hence increased tension generation by facilitating interaction of myosin with F-actin (29). In vitro kinase assays with MYPT as substrate and selectively immunoprecipitated ROCK1 or ROCK2 from lysates of stimulated DC showed that thrombin, PAR1-AP, and, to a lesser extent, PAR3-AP, induced rapid activation of ROCK1 within 1–5 min (Fig. 4*A*). ROCK2 was constitutively activated in nonstimulated LPS-matured DC, which might be due to cell adhesion and engagement of integrins (30). Neither thrombin nor PAR-APs were found to enhance ROCK2 activity (Fig. 4*A*). Thus, in LPS-matured DC thrombin activates ROCK1.

Analysis of MLC2 phosphorylation demonstrated that thrombin and PAR1-AP induce a rapid, time-dependent phosphorylation of MLC2 with similar kinetics (Fig. 4*B*). By contrast, PAR3-AP induced a modest and delayed phosphorylation of MLC2 when compared with thrombin or PAR1-AP (Fig. 4*B*).

In addition to ROCK, members of the MAPK such as p38, JNK, and ERK may also be involved in the process of cell migration by phosphorylating MLC kinase, calpain, or the focal adhesion kinase FAK (31). Activated MLC kinase is able to directly phosphorylate MLC2 (32). Stimulation of LPS-matured DC with thrombin or PAR-APs had no effect on the phosphorylation of p38 (Fig. 4*B*) or JNK (data not shown). However, thrombin and PAR-APs induced rapid activation of ERK1/2, which can contribute to MLC2 phosphorylation and the chemotactic response via activation of the MLC kinase (31). The ERK1/2 activation was induced with kinetics similar to those observed for MLC2 phosphorylation (Fig. 4*B*). The thrombin- and PAR1-AP-induced ERK1/2 activation was selectively inhibited by the specific PAR1 antagonist SCH79797 (22) (Fig. 4*C*).

Activation of ROCK1 and ERK1/2 is indispensable for the PAR-mediated phosphorylation of MLC2 and the DC migration

To analyze the role of ROCK and ERK1/2 activation in the thrombin-induced MLC2 phosphorylation and cell migration, we used inhibitors of ROCK, the ERK pathway and of MLC kinase. The MLC kinase can either be activated by Ca^{2+}/calmodulin and/or by ERK1/2 (29, 33).

The selective ROCK inhibitor Y27632 (34), which targets the ATP-dependent kinase domain (32), abolished the MLC2 phosphorylation induced by thrombin and PAR-APs (Fig. 5*A*), but had no effect on thrombin and PAR1-AP-mediated activation of ERK. Similarly, the ERK pathway inhibitor U0126 (34) effectively inhibited not only ERK1/2 phosphorylation, but also the phosphorylation of MLC2 induced by stimulation with thrombin or PAR1-AP. Inhibition of the MLC kinase with the membrane-permeant inhibitor peptide 18 (35) had no effect on the ERK1/2 phosphorylation, but inhibited the MLC2 phosphorylation, indicating that this kinase is critically involved in the thrombin-induced signaling via ERK1/2 pathway (Fig. 5*A*).

Consistently, the ROCK inhibitor Y27632 as well as the ERK pathway inhibitor U0126 impaired the thrombin- and PAR-AP-induced chemotaxis of mDC, implicating that activation of both kinases is essential for the PAR-mediated chemotactic response (Fig. 5*B*). Similar inhibitory effects on chemotaxis were observed when the MLC kinase inhibitor peptide 18 was used, demonstrating a critical interdependence between the three kinases in terms of DC chemotaxis (Fig. 5*B*).

Stimulation of PAR on LPS-matured DC triggers CCL18/PARC induction

The chemokine CCL18 is synthesized by monocyte-derived DC. Its production in vitro and in vivo by DC is regulated by proinflammatory and anti-inflammatory signals, such as cytokines and PGs (19). Activation of PARs on LPS-matured DC by thrombin, PAR1-AP, and, to a lesser extent, by PAR3-AP induced a markedly increased expression of CCL18 mRNA within 9 h (Fig. 6*A*). Accordingly, mDC stimulated with thrombin, PAR1-APs, or PAR3-AP released increased amounts of CCL18 into the medium (Fig. 6*B*). Pretreatment of LPS-matured DC with inhibitors of ROCK, ERK, or MLC kinase, namely, Y27632, U0126, and peptide 18, showed that ROCK activation is essential for the PAR-mediated expression of CCL18 mRNA and protein in LPS-matured DC (Fig. 6, C and *D*). In contrast to the PAR-induced CCL18 release, the one by unstimulated cells was independent on ROCK activation, because addition of Y27632 (10 μM) to the control cells had no significant effect on the constitutive CCL18 expression (102 $\pm$ 0.3%, $n = 6$). Activation of PARs by thrombin, PAR1-APs, or PAR3-APs did not trigger any release of other

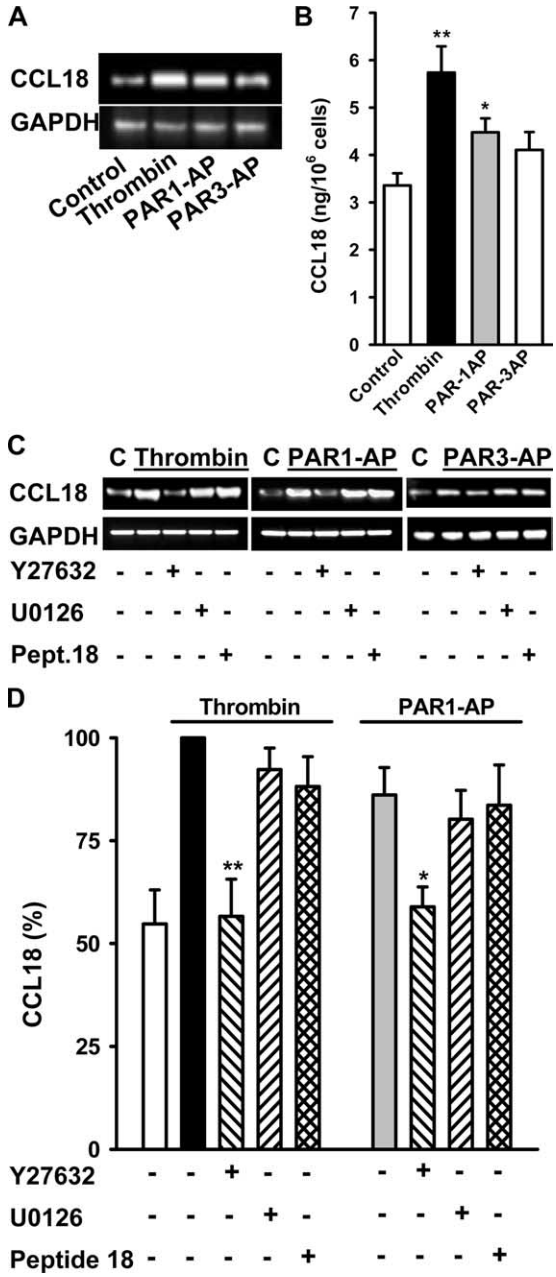

FIGURE 6. Stimulation of thrombin receptors augments production of the chemokine CCL18/PARC. Thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each 100 μM) induce CCL18 mRNA in LPS-matured DC (*A*). Total RNA was isolated after 9 h and subjected to RT-PCR using CCL18-specific primers. GAPDH, control. Thrombin (3 U/ml), PAR1-AP, or PAR3-AP (each 100 μM) induce release of CCL18 from mDC as determined by ELISA after 24 h (*B*). Effects of kinase inhibitors on the CCL18 mRNA expression (*C*). mDC were pretreated for 15 min with the ROCK inhibitor Y27632 or the MEK inhibitor U0126 (each 10 μM) or the MLCK inhibitor peptide 18 (1 μM) and then stimulated with thrombin, PAR1-AP, or PAR3-AP as in *A*. Expression of CCL18 and GAPDH was analyzed by RT-PCR. Effects of the kinase inhibitors on CCL18 release (*D*). mDC were treated as in *C* for 24 h; CCL18 release was analyzed by ELISA. 100% = 5.59 $\pm$ 0.69 ng/ml. All results are representative of three to five experiments; *, $p < 0.05$ and **, $p < 0.01$ vs control.

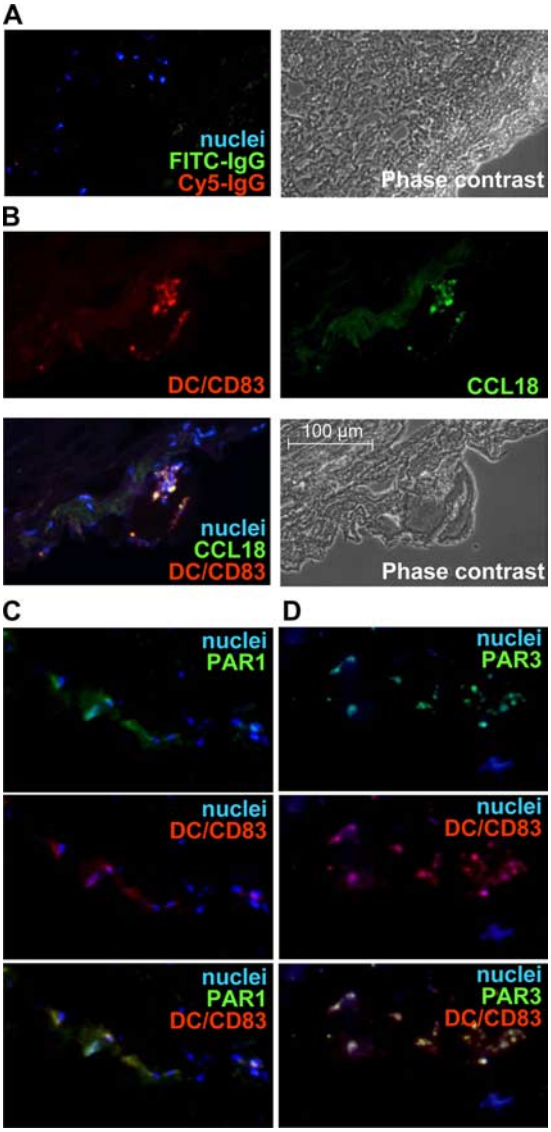

FIGURE 7. Colocalization of CCL18, PAR1, and PAR3 with DC in human atherosclerotic plaque specimens. Cryosections of human atherosclerotic plaques from carotid endarterectomies were stained with anti-CD83 Ab (red), DNA stain Hoechst 33258 (blue), and either anti-CCL18 (*B*), PAR1 (*C*), or PAR3 (*D*) Abs (green). Control sections were stained with fluorescence-labeled control IgG (*A*). Overlay of the stainings revealed colocalization (yellow). Representative sections from nine patients are shown. Original magnification, ×400.

proinflammatory cytokines such as TNF-α, IL-6. or MCP-1 by mDC, nor was the production of IL-10 stimulated (data not shown). Interestingly, real-time PCR analysis demonstrated that thrombin (3 U/ml, 6–24 h) induced in addition to CCL18 expression of CCL22 mRNA (5.78 ± 2.27-fold, 24 h, $n = 4$) and CXCL8 (1.60 ± 0.32-fold, 12 h, $n = 4$), whereas no changes were observed in CCL3, CCL4, and CXCL10 mRNA expression.

CCL18 has no rodent homolog that would allow to study its expression in mouse models (19). We therefore sought to gain evidence for its role in atherosclerotic lesions by multicolor fluorescence microscopy of sections from human atherosclerotic plaques from carotid endarterectomies. Fluorescence microscopy of cryosections immunostained for mature $CD83^+$ DC and CCL18 revealed colocalization by fluorescence overlay of the DC marker CD83 and CCL18, resulting in yellow color (Fig. 7*B*). Similarly, $CD83^+$ DC colocalize in plaques with PAR1 (Fig. 7*C*) and PAR3 (Fig. 7*D*). This indicates that mDC express CCL18 within atherosclerotic plaques, which harbor all factors required for thrombin activation as well as active thrombin (4).

Discussion

The classical paradigm of DC maturation predicts that mDC in contrast to iDC prime T cell responses (8, 20). However, this simple model does not sufficiently reflect the existence of different maturation stages of DC (6, 20). In contrast to other maturation stimuli, only LPS induces development of fully matured DC, which secrete the Th1-polarizing cytokines such as IL-12 (6, 36, 37). Consistent with the diverse response to maturation stimuli, we found differential effects on PAR1 and PAR3 expression in DC. Thus, LPS-matured DC express PAR1 and PAR3, whereas TNF-α- or CD40L-matured DC do not, suggesting that the PAR expression might be important for specific functions confined to LPS-matured DC. As in monocytes (5), neither immature nor mDC express the third thrombin receptor PAR4.

Data on the role of thrombin and its receptors in DC are scarce. We have previously demonstrated that differentiation of human monocytes into iDC results in down-regulation of PAR1, PAR2, and PAR3 (5). On the other hand, serine proteases may well play a role in DC function (38) because administration of bovine thrombin to mice induced autoimmune disease, suggesting an immune stimulatory function possibly mediated by PARs (39, 40). Moreover, administration of the specific thrombin inhibitor hirudin dose-dependently prevented the onset of autoimmune arthritis in mice (41), and thrombin inhibition by melagatran reduces lesion size and promotes plaque stability in $apoE^{-/-}$ mice (42).

Migration is an essential feature of DC that is mainly controlled and coordinated by chemokines (7, 8). It is often assumed that DC migrate only into lymph nodes. However, DC may cluster with T cells within atherosclerotic lesions (9–11, 43). Recently, immunity and DC-centered models of atherogenesis have gained much attention (11, 13). Indeed, the LPS receptor TLR4 is expressed in both human and murine atherosclerotic lesions and TLR4-deficient $apoE^{-/-}$ mice showed a significant reduction of aortic lesion areas establishing a link between TLR4 activation and atherogenesis (15, 17). Moreover, macrophages in atherosclerotic lesions constitutively express tissue factor leading to local generation of thrombin (1, 4). Therefore, thrombin could recruit DC to the vascular-associated lymphoid tissue (11, 43) in atherosclerotic lesions. Indeed, stimulation of the matured DC either with thrombin, PAR1-AP, or PAR3-AP elicited a concentration-dependent chemotaxis. Thrombin binding to PAR1 and PAR3 is potentiated by hirudin-like sequences located C-terminal to the cleavage site enhancing the effective local concentration of thrombin compared with other ligands (1). This might explain why the chemotactic response of mDC to thrombin was higher than that to PAR1-AP.

In addition, the more effective stimulation by thrombin compared with PAR1-AP might also be due to stimulation of two receptors, namely, PAR1 and PAR3. In general, APs serve as convenient tools for dissecting distinct receptor activation. However, receptor activation may be modulated depending on AP cross-reactivity or intermolecular ligation of one PAR molecule by another (1, 44). We used a PAR1-AP that is specific for PAR1 (44). On the contrary, PAR3-AP may not be PAR3 selective. Thus, PAR3 overexpressed in COS7 cells was activated by thrombin, but not by PAR3-AP (1). However, human PAR3-AP stimulate Ca^{2+} and ERK1/2 signaling, as well as proliferation of human vascular muscle cells (26). Only recently it has been proposed that PAR3-AP might be able to activate PAR1 and PAR2 (45). Therefore, it was likely that the small chemotactic response seen with PAR3-AP might have been due to activation of PAR1. In line with this,

PAR1- neutralizing Ab totally inhibited the thrombin-induced chemotaxis. In addition, the specific PAR1 antagonist SCH79797 blocked the ERK1/2 phosphorylation induced by thrombin, indicating signaling primarily via PAR1.

Chemotaxis requires integration of various signaling pathways, including small Rho GTPases and MAPK (29, 31). Ligated PAR1 can activate heterotrimeric G proteins from different families, such as $G_{12/13}$, G_q, and G_i, which are all expressed in DC (46) and which can signal independent from of each other (1). Whereas G_q mediates calcium signaling, $G_{12/13}$ is coupled to Rho/ROCK (1, 27) and G_i is linked to the Ras/ERK pathway (47). These multiple signaling pathways can be activated in endothelial cells, where PAR1 modulates several independent functional responses, such as endothelial permeability via $G_{12/13}$/Rho, intracellular calcium increase via G_q, and ERK activation through G_i/Ras-MEK1 pathways (27, 47).

Because thrombin stimulation did not trigger any intracellular Ca^{2+} response in LPS-matured DC, we further analyzed activation of Rho/ROCK and ERK signaling pathways.

Interaction of myosin with actin, essential for cell movement, is regulated primarily by phosphorylation of the regulatory myosin L chain MLC2. MLC2 phosphorylation, in turn, is regulated by the Ca^{2+}/calmodulin-regulated MLC kinase. In addition, activated ERK is able to phosphorylate and activate MLC kinase directly (31). Accordingly, constitutively active MEK1, an upstream kinase of ERK, induces MLC kinase activation, MLC2 phosphorylation, and cell migration (33). Interestingly, this process occurs without an intracellular Ca^{2+} concentration increase. Thrombin and PAR-APs induced a rapid, time-dependent activation of ERK in DC, the kinetics of which were similar to those of MLC2 phosphorylation, suggesting that ERK1/2 mediates phosphorylation of the regulatory MLC. Indeed, inhibition of ERK activity by U0126 abolished MLC2 phosphorylation and thrombin-induced DC migration, confirming a central role of ERK in MLC2 phosphorylation.

Besides ERK and MLC kinase, also Rho GTPases are involved in the regulation of myosin phosphorylation through the downstream effectors ROCKs. ROCK phosphorylates the myosin-binding subunit of MLC phosphatase, leading to its inhibition and the subsequent promotion of the MLC2 phosphorylation associated with contraction without intracellular Ca^{2+} concentration increases (48). Accordingly, dominant negative ROCK or treatment with ROCK inhibitors led to enhanced MLC phosphatase activity and increased MLC2 dephosphorylation in migrating cells (49). Likewise, in monocytes Rho and its downstream effector ROCK, but not Rac1 or Cdc42, are essential for cell migration (50). Inhibition of ROCK activity in DC totally inhibited MLC2 phosphorylation, but had no effect on ERK phosphorylation induced by thrombin. Hence, ROCK contributes to increased MLC2 phosphorylation via inactivation of MLC phosphatase. Thus, the coordinated activation of Rho/ROCK1 and ERK/MLC kinase pathways is indispensable for the thrombin-induced chemotaxis of DC.

Interestingly, in DC thrombin stimulated only one of the two Rho kinases, namely, ROCK1. The finding that thrombin triggers chemotaxis in mDC through selective activation of ROCK1 is consistent with data showing that in fibroblasts ROCK1 rather than ROCK2 is important for stress fiber formation, whereas ROCK2 is involved in phagocytosis (51).

Whereas a role for Rho/ROCK in chemotaxis is well established, recent findings indicate that Rho might also modulate transcription factor activity and subsequent gene induction (29), e.g., expression of vascular endothelial growth factor (52). Activation of transcription factors might also induce cytokines and chemokines required for DC function in adaptive immunity (7). Treatment of DC with thrombin, PAR1-APs, and PAR3-APs induced expression of the CC chemokine CCL18/PARC, a potent chemoattractant for T cells and iDC (19). CCL18 is crucial for the homeostatic trafficking of lymphocytes and DC to tissues where CCL18 is constitutively expressed (19). CCL18 seems to be associated with a number of pathological conditions including malignances and chronic inflammation (19). Moreover, CCL18 expression is also detected in atherosclerotic lesions (21). In monocytes in vitro its production is induced by LPS and CD40L (53), whereas in iDC it is activated by anti-inflammatory stimuli such as IL-10 or vitamin D_3 (54, 55). Since rodents do not have a CCL18 analog, studies on its regulation and function in vivo have been hampered (19). Our data show that mature $CD83^+$ DC colocalize with CCL18 within human atherosclerotic plaques and that thrombin potentiates expression of CCL18 in LPS-matured DC via the Rho/ROCK-dependent pathway, whereas it does not induce release of T cell-activating proinflammatory cytokines such as TNF-α, IL-6, and the CC chemokine CCL2.

In conclusion, our data demonstrate expression of functionally active thrombin receptors on LPS-matured DC. In these cells, thrombin was identified as a potent chemoattractant and as a CCL18 chemokine inducer signaling via Rho/ERK-dependent pathways. Taking into account the presence of thrombin and PAR-expressing DC in atherosclerotic lesions, this mechanism of DC activation provides a novel link between immunity and atherogenesis.

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Disclosures

The authors have no financial conflict of interest.

References

1. Coughlin, S. R. 2005. Protease-activated receptors in hemostasis, thrombosis, and vascular biology. *J. Thromb. Haemost.* 3: 1800–1814.
2. Hou, L., G. L. Howells, S. Kapas, and M. G. Macey. 1998. The protease-activated receptors and their cellular expression and function in blood-related cells. *Br. J. Haematol.* 101: 1–9.
3. Smith, E. B., L. Crosbie, and S. Carey. 1996. Prothrombin-related antigens in human aortic intima. *Semin. Thromb. Hemostasis* 22: 347–350.
4. Westmuckett, A. D., C. Lupu, D. A. Goulding, S. Das, V. V. Kakkar, and F. Lupu. 2000. In situ analysis of tissue factor-dependent thrombin generation in human atherosclerotic vessels. *Thromb. Haemost.* 84: 904–911.
5. Colognato, R., J. R. Slupsky, M. Jendrach, L. Burysek, T. Syrovets, and T. Simmet. 2003. Differential expression and regulation of protease-activated receptors in human peripheral monocytes and monocyte-derived antigen-presenting cells. *Blood* 102: 2645–2652.
6. Gad, M., M. H. Claesson, and A. E. Pedersen. 2003. Dendritic cells in peripheral tolerance and immunity. *APMIS* 111: 766–775.
7. Steinman, R. M., and H. Hemmi. 2006. Dendritic cells: translating innate to adaptive immunity. *Curr. Top. Microbiol. Immunol.* 311: 17–58.
8. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18: 767–811.
9. Bobryshev, Y. V. 2005. Dendritic cells in atherosclerosis: current status of the problem and clinical relevance. *Eur. Heart J.* 26: 1700–1704.
10. Millonig, G., H. Niederegger, W. Rabl, B. W. Hochleitner, D. Hoefer, N. Romani, and G. Wick. 2001. Network of vascular-associated dendritic cells in intima of healthy young individuals. *Arterioscler. Thromb. Vasc. Biol.* 21: 503–508.
11. Wick, G., M. Knoflach, and Q. Xu. 2004. Autoimmune and inflammatory mechanisms in atherosclerosis. *Annu. Rev. Immunol.* 22: 361–403.
12. Jongstra-Bilen, J., M. Haidari, S. N. Zhu, M. Chen, D. Guha, and M. I. Cybulsky. 2006. Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *J. Exp. Med.* 203: 2073–2083.
13. Doherty, T. M., E. A. Fisher, and M. Arditi. 2006. TLR signaling and trapped vascular dendritic cells in the development of atherosclerosis. *Trends Immunol.* 27: 222–227.
14. Yilmaz, A., M. Lochno, F. Traeg, I. Cicha, C. Reiss, C. Stumpf, D. Raaz, T. Anger, K. Amann, T. Probst, et al. 2004. Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. *Atherosclerosis* 176: 101–110.
15. Michelsen, K. S., M. H. Wong, P. K. Shah, W. Zhang, J. Yano, T. M. Doherty, S. Akira, T. B. Rajavashisth, and M. Arditi. 2004. Lack of Toll-like receptor 4 or

myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc. Natl. Acad. Sci. USA* 101: 10679–10684.
16. Walton, K. A., X. Hsieh, N. Gharavi, S. Wang, G. Wang, M. Yeh, A. L. Cole, and J. A. Berliner. 2003. Receptors involved in the oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8: a role for Toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein. *J. Biol. Chem.* 278: 29661–29666.
17. Bjorkbacka, H. 2006. Multiple roles of Toll-like receptor signaling in atherosclerosis. *Curr. Opin. Lipidol.* 17: 527–533.
18. Fields, R. C., J. G. Schoenecker, J. P. Hart, M. R. Hoffman, S. V. Pizzo, and J. H. Lawson. 2003. Protease-activated receptor-2 signaling triggers dendritic cell development. *Am. J. Pathol.* 162: 1817–1822.
19. Schutyser, E., A. Richmond, and J. Van Damme. 2005. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J. Leukocyte Biol.* 78: 14–26.
20. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat. Rev. Immunol.* 6: 476–483.
21. Reape, T. J., K. Rayner, C. D. Manning, A. N. Gee, M. S. Barnette, K. G. Burnand, and P. H. Groot. 1999. Expression and cellular localization of the CC chemokines PARC and ELC in human atherosclerotic plaques. *Am. J. Pathol.* 154: 365–374.
22. Ahn, H. S., C. Foster, G. Boykow, A. Stamford, M. Manna, and M. Graziano. 2000. Inhibition of cellular action of thrombin by *N*-3-cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7*H*-pyrrolo[3,2-f]quinazoline-1,3-diamine] (SCH 79797), a nonpeptide thrombin receptor antagonist. *Biochem. Pharmacol.* 60: 1425–1434.
23. Yang, D., Q. Chen, B. Gertz, R. He, M. Phulsuksombati, R. D. Ye, and J. J. Oppenheim. 2002. Human dendritic cells express functional formyl peptide receptor-like-2 (FPRL2) throughout maturation. *J. Leukocyte Biol.* 72: 598–607.
24. Syrovets, T., M. Jendrach, A. Rohwedder, A. Schüle, and T. Simmet. 2001. Plasmin-induced expression of cytokines and tissue factor in human monocytes involves AP-1 and IKKβ-mediated NF-κB activation. *Blood* 97: 3941–3950.
25. Syrovets, T., B. Tippler, M. Rieks, and T. Simmet. 1997. Plasmin is a potent and specific chemoattractant for human peripheral monocytes acting via a cyclic guanosine monophosphate-dependent pathway. *Blood* 89: 4574–4583.
26. Bretschneider, E., R. Spanbroek, K. Lotzer, A. J. Habenicht, and K. Schrör. 2003. Evidence for functionally active protease-activated receptor-3 (PAR-3) in human vascular smooth muscle cells. *Thromb. Haemost.* 90: 704–709.
27. McLaughlin, J. N., L. Shen, M. Holinstat, J. D. Brooks, E. Dibenedetto, and H. E. Hamm. 2005. Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J. Biol. Chem.* 280: 25048–25059.
28. Affolter, M., and C. J. Weijer. 2005. Signaling to cytoskeletal dynamics during chemotaxis. *Dev. Cell* 9: 19–34.
29. Riento, K., and A. J. Ridley. 2003. Rocks: multifunctional kinases in cell behaviour. *Nat. Rev. Mol. Cell Biol.* 4: 446–456.
30. Cox, E. A., S. K. Sastry, and A. Huttenlocher. 2001. Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol. Biol. Cell* 12: 265–277.
31. Huang, C., K. Jacobson, and M. D. Schaller. 2004. MAP kinases and cell migration. *J. Cell Sci.* 117: 4619–4628.
32. Noma, K., N. Oyama, and J. K. Liao. 2006. Physiological role of ROCKs in the cardiovascular system. *Am. J. Physiol.* 290: C661–C668.
33. Klemke, R. L., S. Cai, A. L. Giannini, P. J. Gallagher, P. de Lanerolle, and D. A. Cheresh. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137: 481–492.
34. Davies, S. P., H. Reddy, M. Caivano, and P. Cohen. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 351: 95–105.
35. Clayburgh, D. R., T. A. Barrett, Y. Tang, J. B. Meddings, L. J. Van Eldik, D. M. Watterson, L. L. Clarke, R. J. Mrsny, and J. R. Turner. 2005. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. *J. Clin. Invest.* 115: 2702–2715.
36. Morelli, A. E., A. F. Zahorchak, A. T. Larregina, B. L. Colvin, A. J. Logar, T. Takayama, L. D. Falo, and A. W. Thomson. 2001. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood* 98: 1512–1523.
37. Rivas-Carvalho, A., M. A. Meraz-Rios, L. Santos-Argumedo, S. Bajana, G. Soldevila, M. E. Moreno-Garcia, and C. Sanchez-Torres. 2004. CD16$^+$ human monocyte-derived dendritic cells matured with different and unrelated stimuli promote similar allogeneic Th2 responses: regulation by pro- and anti-inflammatory cytokines. *Int. Immunol.* 16: 1251–1263.
38. Santambrogio, L., A. K. Sato, G. J. Carven, S. L. Belyanskaya, J. L. Strominger, and L. J. Stern. 1999. Extracellular antigen processing and presentation by immature dendritic cells. *Proc. Natl. Acad. Sci. USA* 96: 15056–15061.
39. Cronstein, B. N. 2003. Bovine thrombin and systemic autoimmunity. *Am. J. Pathol.* 162: 1389.
40. Schoenecker, J. G., R. K. Johnson, A. P. Lesher, J. D. Day, S. D. Love, M. R. Hoffman, T. L. Ortel, W. Parker, and J. H. Lawson. 2001. Exposure of mice to topical bovine thrombin induces systemic autoimmunity. *Am. J. Pathol.* 159: 1957–1969.
41. Marty, I., V. Peclat, G. Kirdaite, R. Salvi, A. So, and N. Busso. 2001. Amelioration of collagen-induced arthritis by thrombin inhibition. *J. Clin. Invest.* 107: 631–640.
42. Bea, F., J. Kreuzer, M. Preusch, S. Schaab, B. Isermann, M. E. Rosenfeld, H. Katus, and E. Blessing. 2006. Melagatran reduces advanced atherosclerotic lesion size and may promote plaque stability in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 26: 2787–2792.
43. Wick, G., M. Romen, A. Amberger, B. Metzler, M. Mayr, G. Falkensammer, and Q. Xu. 1997. Atherosclerosis, autoimmunity, and vascular-associated lymphoid tissue. *FASEB J.* 11: 1199–1207.
44. Blackhart, B. D., K. Emilsson, D. Nguyen, W. Teng, A. J. Martelli, S. Nystedt, J. Sundelin, and R. M. Scarborough. 1996. Ligand cross-reactivity within the protease-activated receptor family. *J. Biol. Chem.* 271: 16466–16471.
45. Hansen, K. K., M. Saifeddine, and M. D. Hollenberg. 2004. Tethered ligand-derived peptides of proteinase-activated receptor 3 (PAR3) activate PAR1 and PAR2 in Jurkat T cells. *Immunology* 112: 183–190.
46. Shi, G. X., K. Harrison, S. B. Han, C. Moratz, and J. H. Kehrl. 2004. Toll-like receptor signaling alters the expression of regulator of G protein signaling proteins in dendritic cells: implications for G protein-coupled receptor signaling. *J. Immunol.* 172: 5175–5184.
47. Ellis, C. A., A. B. Malik, A. Gilchrist, H. Hamm, R. Sandoval, T. Voyno-Yasenetskaya, and C. Tiruppathi. 1999. Thrombin induces proteinase-activated receptor-1 gene expression in endothelial cells via activation of G_i-linked Ras/mitogen-activated protein kinase pathway. *J. Biol. Chem.* 274: 13718–13727.
48. van Nieuw Amerongen, G. P., and V. W. van Hinsbergh. 2001. Cytoskeletal effects of Rho-like small guanine nucleotide-binding proteins in the vascular system. *Arterioscler. Thromb. Vasc. Biol.* 21: 300–311.
49. Kawano, Y., Y. Fukata, N. Oshiro, M. Amano, T. Nakamura, M. Ito, F. Matsumura, M. Inagaki, and K. Kaibuchi. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J. Cell Biol.* 147: 1023–1038.
50. Honing, H., T. K. van den Berg, S. M. van der Pol, C. D. Dijkstra, R. A. van der Kammen, J. G. Collard, and H. E. de Vries. 2004. RhoA activation promotes transendothelial migration of monocytes via ROCK. *J. Leukocyte Biol.* 75: 523–528.
51. Yoneda, A., H. A. Multhaupt, and J. R. Couchman. 2005. The Rho kinases I and II regulate different aspects of myosin II activity. *J. Cell Biol.* 170: 443–453.
52. Mizukami, Y., K. Fujiki, E. M. Duerr, M. Gala, W. S. Jo, X. Zhang, and D. C. Chung. 2006. Hypoxic regulation of vascular endothelial growth factor through the induction of phosphatidylinositol 3-kinase/Rho/ROCK and c-Myc. *J. Biol. Chem.* 281: 13957–13963.
53. Pivarcsi, A., M. Gombert, M. C. Dieu-Nosjean, A. Lauerma, R. Kubitza, S. Meller, J. Rieker, A. Muller, L. Da Cunha, A. Haahtela, et al. 2004. CC chemokine ligand 18, an atopic dermatitis-associated and dendritic cell-derived chemokine, is regulated by staphylococcal products and allergen exposure. *J. Immunol.* 173: 5810–5817.
54. Vulcano, M., S. Struyf, P. Scapini, M. Cassatella, S. Bernasconi, R. Bonecchi, A. Calleri, G. Penna, L. Adorini, W. Luini, et al. 2003. Unique regulation of CCL18 production by maturing dendritic cells. *J. Immunol.* 170: 3843–3849.
55. van Lieshout, A. W., T. R. van der Voort, L. M. P. le Blanc, M. F. Roelofs, B. W. Schreurs, P. L. van Riel, G. J. Adema, and T. R. Radstake. 2006. Novel insights in the regulation of CCL18 secretion by monocytes and dendritic cells via cytokines, Toll-like receptors and rheumatoid synovial fluid. *BMC Immunol.* 7: 23.