The soluble D2D3$_{88-274}$ fragment of the urokinase receptor inhibits monocyte chemotaxis and integrin-dependent cell adhesion

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Summary

We have previously shown that chymotrypsin-cleaved soluble uPAR (D2D3$_{88-274}$) elicits migration of monocytic cells through interaction with FPRL-1, a G protein-coupled receptor that is homologous to the fMLP receptor. Here, we report that D2D3$_{88-274}$ also modulates the ability of monocytes to migrate in response to other chemokines. Pretreatment of monocytes with increasing amounts of D2D3$_{88-274}$ prevents cell migration in response to MCP-1, RANTES and fMLP. We demonstrate that D2D3$_{88-274}$ does not inhibit MCP-1 receptor binding, elicit CCR2 internalization and prevent MCP-1-induced intracellular Ca^{2+} increase. Thus, CCR2 receptor desensitization cannot account for D2D3$_{88-274}$-mediated inhibition of MCP-1-induced cell migration. Rather, we show that pretreatment of monocytes with D2D3$_{88-274}$ dramatically decreases chemokine-induced integrin-dependent rapid cell adhesion by interacting with FPRL-1. Together, our results indicate that chemokine-dependent cell migration can be regulated not only by homologous and heterologous receptor desensitization, but also by inhibition of integrin-dependent cell adhesion, an important step in cell transmigration.

Key words: Monocytes, Chemoattractants, Cell migration, Cell adhesion

Introduction

Cell migration across the blood barrier and into tissues is one of the first events during inflammation, immune response against infection and tissue remodeling following injury. Migration is a complex response that requires the coordinated cooperation of multiple cell surface receptors, including chemoattractant receptors that direct oriented migratory signals, adhesion receptors that modulate interactions of migrating cells with the endothelium and with the extracellular matrix, and protease receptors that mediate interactions of cells with their extracellular environment (Pluskota et al., 2003). Monocytes undergo a complex sequence of adhesive and locomotive steps as they are recruited to migrate from the vascular space into sites of inflammation. Extravasation is usually preceded by a phase of loose adhesion (tethering and rolling), followed by a phase of firm attachment mediated by β1 integrin and β2 integrin (Beekhuizen and van Furth, 1993). The binding capacity of β2 integrin is mostly regulated by an inside-out signaling mechanism (Stewart and Hogg, 1996). These signals determine the rapid transition between low and high heterodimer affinity states during the sequential attachment and detachment steps that are necessary for cell recruitment and motility (Laudanna et al., 2002). Chemokines modulate monocyte integrin affinity within fractions of seconds, which results either in augmented reversible adhesions or immediate arrest on the vascular endothelium (Alon and Feigelson, 2002).

A growing number of soluble factors are now known to elicit migration of monocytes. Among these, monocyte chemotactic protein (MCP-1) is the best characterized. MCP-1 elicits monocyte recruitment to inflamed sites by promoting integrin-dependent cell adhesion and by inducing migration across the vessel wall (Ashida et al., 2001). In addition to MCP-1, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) and formyl-methyonyl-leucyl-proline (fMLP) have been described to elicit monocyte migration in vivo (Fine et al., 2001). We previously reported that, in addition to these chemokines, chymotrypsin-cleaved soluble urokinase-type plasminogen activator receptor (uPAR) (D2D3$_{88-274}$) elicits migration of monocytes and other cell types (Blasi and Carmeliet, 2002; Fazioli et al., 1997; Resnati et al., 1996; Resnati et al., 2002). This occurs via the interaction of uPAR-derived D2D3$_{88-274}$ with FPRL-1, a G protein-coupled receptor homologue of the fMLP receptor.

D2D3$_{88-274}$ induced cell migration is sensitive to pertussis toxin and involves phosphorylation of p56-59^{hck} tyrosine kinase (Bohuslav et al., 1995; Resnati et al., 1996) and activation of the extracellular signal-regulated kinases ERK1 and 2 (ERK1/2) (Degryse et al., 2001). The chemotactic activity of D2D3$_{88-274}$, together with the finding that the cleaved form of uPAR is naturally produced and found in tissues and in biological fluids (Sidenius et al., 2000), suggests that D2D3$_{88-274}$ plays an important role during inflammatory

reactions and during the pathophysiology of many diseases. We therefore investigated whether $D2D3_{88-274}$ interferes with chemokine-induced cell migration. Here we show that pretreatment of monocytes with $D2D3_{88-274}$ prevents the ability of the cells to migrate in response to MCP-1. This effect is not because of classical mechanisms of chemokine receptor desensitization, but rather because of the interference of $D2D3_{88-274}$ with chemokine-induced rapid integrin activation and dependent cell adhesion. We suggest that interference with β2-integrin-rapid adhesion should be considered as a new mechanism of chemoattractant receptor cross regulation.

Materials and Methods

Cells and reagents

THP-1 cells (American Type Culture Collection, Rockville, MD) were grown in suspension in RPMI medium (GIBCO/BRL) supplemented with L-glutamine, penicillin/streptomycin and 10% fetal bovine serum (FBS) (GIBCO/BRL). Human peripheral blood monocytes were isolated from buffy coats of healthy volunteers. Briefly, mononuclear cells were enriched by two rounds of centrifugation (460 *g*) and purified over sequential Ficoll™ and Percoll™ (Amersham Pharmacia) gradients. The purity of the obtained population was routinely verified by cytofluorimetric analysis following the staining of the cells with anti-CD14 monoclonal antibody (mAb) (Pharmingen). Events were acquired by FACScan™ (Becton Dickinson) and analyzed using CELLQuest Software. Generally, 95% of the cells stained positive for CD14.

$D1_{1-87}$ (referred as to D1 throughout the manuscript) and $D2D3_{88-274}$ fragments were obtained from chymotrypsin-cleaved uPAR that was purified from the conditioned medium of stably transfected cell lines as previously described (Resnati et al., 1996). A recombinant soluble form of uPAR containing the chemotactic region ($D2D3_{84-274}$) was generated by PCR and subcloned into the *Nco*I-*Eco*RI sites of the pBNSEN eukaryotic expression vector (Pallisgaard et al., 1994). $D2D3_{84-274}$ and $D2D3_{92-274}$ [the recombinant soluble form of uPAR missing the chemotactic region (ΔD2D3)] was purified from conditioned medium of transiently transfected 293-cells as previously described (Fazioli et al., 1997). Chemotactic peptide fMLP was purchased from Sigma. MCP-1 was obtained from PeproTech EC. The MMK-1 peptide has been previously described (Klein et al., 1998). The anti-FPRL-1 polyclonal antibody (Ab) (N77) was generated by immunizing rabbits with an formyl peptide receptor-like 1 (FPRL-1)-derived peptide (ASWGGTPEERLKC) coupled to keyhole limpet hemocyanin (KLH), and purified by affinity chromatography on CNBr-peptide linked beads. The Ab was then tested in flow cytometry analysis and shown capable of recognizing FPRL-1 expressed by transfected cells. Furthermore, the N77 Ab inhibited MMK-induced cell migration (M.R., unpublished data). 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2), an inhibitor of the Src family of tyrosine kinases, was purchased from Calbiochem. PD-098059 and SB-203580, selective inhibitors of the mitogen activated protein kinase (MAPK) and p38 respectively, were purchased from Sigma.

Chemotaxis assay

Migration of THP-1 cells or monocytes was assessed using a 48-well microchemotaxis Boyden chamber (Neuroprobe, Cabin John, MD) as previously described (Resnati et al., 1996). Briefly, different concentrations of the appropriate chemoattractant diluted in serum-free RPMI medium were placed in the lower compartment of the Boyden chamber. The cells (5×10^4 cells in 50 μl medium) were added to the upper compartment and allowed to migrate through an uncoated filter (pore size 5 μm) placed between the two compartments. After a 90-minute incubation at 37°C in humidified air with 5% CO_2, the filter

was removed, scraped, fixed and stained with Diff-Quik (Dade Diagnostics, Aguada, PR). Migrated cells were counted by light microscopy in a high-power field. Results, expressed as the mean±s.d. from triplicate samples, are representative of at least three experiments. Migration in the absence of chemoattractant was set as 100%. The chemotactic index is given by the number of the cells migrated in the presence versus those migrated in the absence of chemoattractant. In our experiments, cells were pretreated with the reagents as indicated in the respective figures for 15-20 minutes at 37°C, and were then allowed to migrate in the Boydon chamber. Reagents used for the pretreatment were present during the assay. The ability of the cells to migrate was determined as described above (Rabbani and Gladu, 2002). Migration of untreated cells was set to 100%.

THP-1 cells were incubated for 15 minutes with either anti-β2-integrin (clone IB4) or an isotype-matched control-Ab at a concentration of 15 μg/ml (as indicated).

Binding assay

^{125}I-MCP-1 (1 nM) (Amersham) was incubated with $1.5-2 \times 10^6$ cells in 100 μl of binding buffer (RPMI medium 1640, 0.5% BSA) in the presence of increasing concentrations of unlabeled MCP-1, $D2D3_{88-274}$ and D1 for 4 hours at 4°C. Thereafter, the cells were washed once with 1 ml of RPMI medium 1640, 0.5% BSA and centrifuged through a 10% sucrose-PBS cushion in Eppendorf tubes. The pellet-containing tips of the tubes were cut off and counted in a gamma counter.

Measurement of intracellular Ca^{2+} concentration

Cells were washed, resuspended in Krebs-Ringer-HEPES-glucose-glutamine buffer (KRH buffer) and incubated with 1 mM FURA-2 (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Fluorescence output was measured in a luminescence spectrometer (Perkin Elmer, LS50B) at 340 nm and the relative ratio of fluorescence emitted at 500 nm was continuously recorded.

Determination of F-actin

Cells were washed twice with PBS and resuspended in serum-free RPMI medium 1640. Cells were than treated by adding 10 nM $D2D3_{88-274}$, 10 nM urokinase-type plasminogen activator (uPA) or medium for 30 minutes and stimulated with 100 nM MCP-1 without removing the original medium at the different time points. At the end of the stimulation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Fixed cells were stained with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) for 15 minutes on ice. The amount of bound FITC-labeled phalloidin was than evaluated by flow-cytometry analysis.

Rapid adhesion assay

Human monocytes were washed and resuspended in PBS with 1 mM $CaCl_2$, $MgCl_2$ and 10% FCS (at a concentration of 4×10^6 per ml). Fibrinogen was used to coat 18-well (5 mm diameter) Teflon-coated glass slides (Cel-Line) at 4°C (Berlin et al., 1993; Campbell et al., 1996). After overnight incubation, fibrinogen was removed from the slides by suction and replaced with 20 μl of the cell suspension (8×10^4 cells), which was allowed to settle for 5 minutes. After settling, 5 μl of a 5× solution of the indicated chemokine were added to the cell. After 3 minutes the slides were washed by dipping in PBS and then fixed in 1.5% of glutaraldehyde. Where indicated, the cells were pretreated with the reagents indicated in the figures for 15-20 minutes at 37°C, and then allowed adhere to fibrinogen-coated glasses. Reagents used for the pretreatment were present during the assay. In some experiments, monocytes were first incubated with inhibitors of intracellular signaling for 30 minutes at 37°C, and then incubated with

D2D3$_{88-274}$ for an additional 20 minutes at 37°C. Thereafter, adhesion was determined as described above. The number of adherent cells was counted over at least four fields (0.2 mm) using video-imaging software (NIH Imagine) (Laudanna et al., 1997). Experiments were routinely performed in triplicate.

Results

D2D3$_{88-274}$ inhibits MCP-1-induced monocyte migration

To further characterize the chemotactic ability of D2D3$_{88-274}$, we investigated whether this molecule interferes with chemokine-induced monocyte migration. Soluble D1 and D2D3$_{88-274}$ fragments were obtained from chymotrypsin cleaved uPAR as previously described (Fazioli et al., 1997; Resnati et al., 1996). Recombinant soluble forms of uPAR that either contain or lack the minimal chemotactic epitope at amino acid positions 88-92 (D2D3$_{92-274}$ and ΔD2D3, respectively) were purified from conditioned medium of transiently transfected 293-cells. As expected, D2D3$_{88-274}$, which contains the minimal chemotactic epitope 88-92, and not D1, which lacks the chemotactic sequence, elicited migration of human THP-1 monocytic cells (Fazioli et al., 1997) and of peripheral blood-derived human monocytes (Fig. 1A). Similarly to D1, ΔD2D3 also failed to elicit migration of monocytes through the Boyden chamber filter. These results confirmed that also for human monocytes, the chemotactic fragment of soluble uPAR is mapped in the D1-D2D3 linker region. We next asked whether, as previously shown for other chemoattractants and for a number of chemokines (Olson and Ley, 2002), D2D3$_{88-274}$ interferes with the activity of other pro-inflammatory stimuli. Monocytes (Fig. 1B) and THP-1 cells (Fig. 1C) were pretreated for 15 minutes with D2D3$_{88-274}$, D1 or ΔD2D3 at the concentrations indicated in the figure and the cells were subsequently allowed to migrate in response to MCP-1. Cells pretreated with D1 or ΔD2D3 migrated as the control cells in response to the chemokine, whereas the migration of cells pretreated with D2D3$_{88-274}$ was reduced by up to 65%. Similar results were obtained by using recombinant soluble D2D3$_{84-274}$, eliminating the possibility that the effect is owing to chymotrypsin contamination.

D2D3$_{88-274}$ does not inhibit MCP-1/CCR2 binding or signaling

We reasoned that D2D3$_{88-274}$ might inhibit MCP-1-induced cell migration by competing for MCP-1 binding to CCR2, by inducing CCR2 receptor downregulation or by eliciting CCR2 desensitization. Initially we investigated whether D2D3$_{88-274}$ directly binds to CCR2. For this, iodinated D2D3$_{88-274}$ was used in binding assays performed on parental and CCR2-transfected CHO cells, and showed that iodinated D2D3$_{88-274}$ did not bind to any parental CCR2-expressing cells (data not shown). We then investigated whether D2D3$_{88-274}$ competes for MCP-1 binding to its receptor. To this aim, binding assays with iodinated MCP-1 were performed on monocytes (Fig. 2A), THP-1 cells (not shown) and CCR2-expressing CHO cells (Fig. 2B) in the absence and in the presence of an excess of unlabeled competitors. As expected, unlabeled MCP-1 inhibited up to 50% of ^{125}I-MCP-1 binding to human monocytes (Dzenko et al., 2001) and up to 88% on CCR2-CHO cells. By contrast, both D2D3$_{88-274}$ and D1 were completely
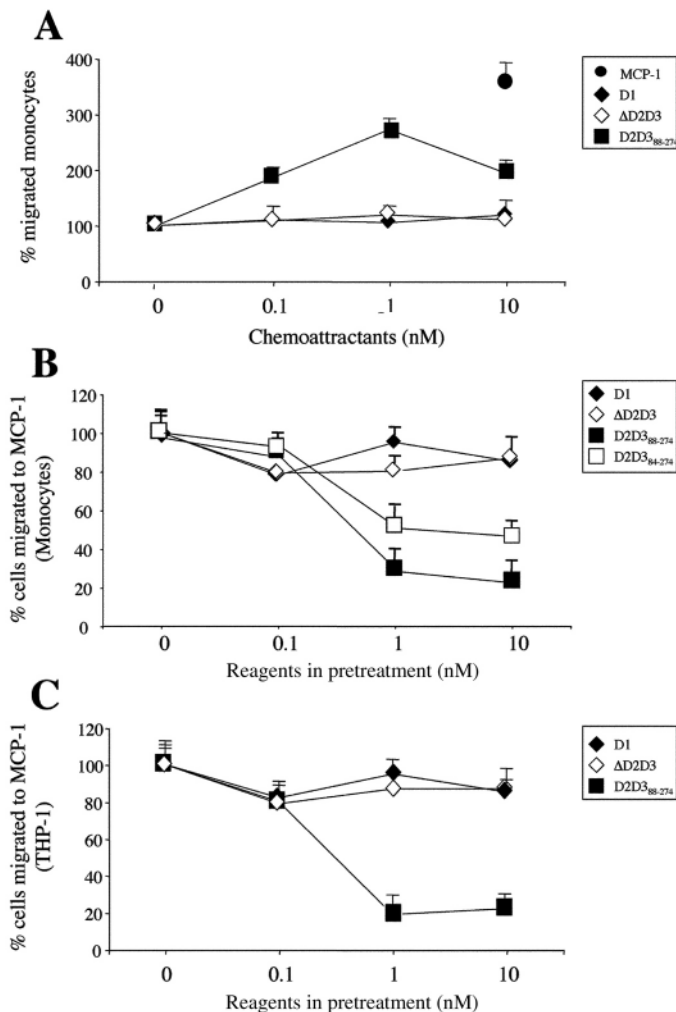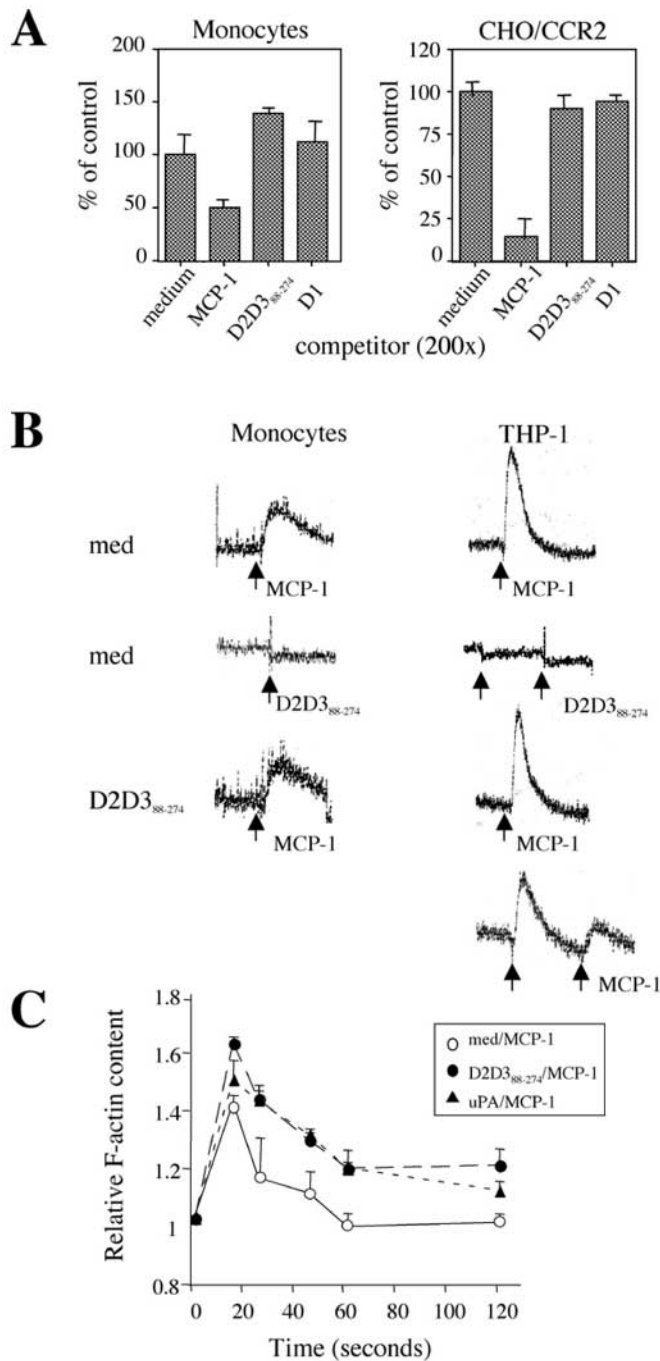

Fig. 1. D2D3$_{88-274}$ inhibits MCP-1 induced monocyte migration. (A) Human monocytes were obtained from the peripheral blood of healthy volunteers using Ficoll and Percoll gradients. Cell migration in response to the indicated concentration of D2D3$_{88-274}$ (■), ΔD2D3 (◇) and D1 (◆) was determined in a Boyden chamber as described in Materials and Methods. As control, cells were allowed to migrate in response to 2 nM MCP-1 (●). The number of cells migrating in the absence of chemoattractant was taken as 100%. (B) Monocytes or (C) THP-1 cells were left either untreated or stimulated for 15 minutes with D2D3$_{88-274}$ (■), D2D3$_{84-274}$ (□), ΔD2D3 (◇) and D1 (◆) as indicated. Thereafter, the ability of the cells to migrate in response to 2 nM MCP-1 was determined. The number of cells migrating towards MCP-1 was taken as 100% and the values of cells migrating after pretreatment with D2D3$_{88-274}$ were calculated relative to this. Each point represents the mean±s.d. of three independent experiments.

ineffective in preventing MCP-1 binding. In some experiments, cells were treated from 2 to 24 hours with either unlabeled MCP-1 or D2D3$_{88-274}$. Again, while variable degrees of inhibition were observed with MCP-1 pretreatments [consistent with receptor internalization, (Sarau et al., 1997)], no effect was observed with D2D3$_{88-274}$ or D1. These data indicate that inhibition of MCP-1/CCR2 interaction cannot account for D2D3$_{88-274}$-dependent inhibition of MCP-1-induced cell migration.

We next investigated whether D2D3$_{88-274}$ prevents MCP-1-induced Ca^{2+} flux, one of the earliest events induced by MCP-1 binding to its receptor (Gerszten et al., 1999). Monocytes and THP-1 cells were loaded with FURA-2 and changes in intracellular Ca^{2+} concentration following the stimulation with either D2D3$_{88-274}$ or MCP-1 were measured by fluorimetric analysis. Stimulation of the cells with D2D3$_{88-274}$ did not induce any detectable change in the Ca^{2+} concentration, whereas MCP-1 induced a transient Ca^{2+} increase (Fig. 2B). Cells were then pretreated with D2D3$_{88-274}$, loaded with FURA-2 and stimulated with MCP-1. The results reported in Fig. 2B indicate that untreated and treated monocytes and THP-1 cells respond to a similar extent to MCP-1.
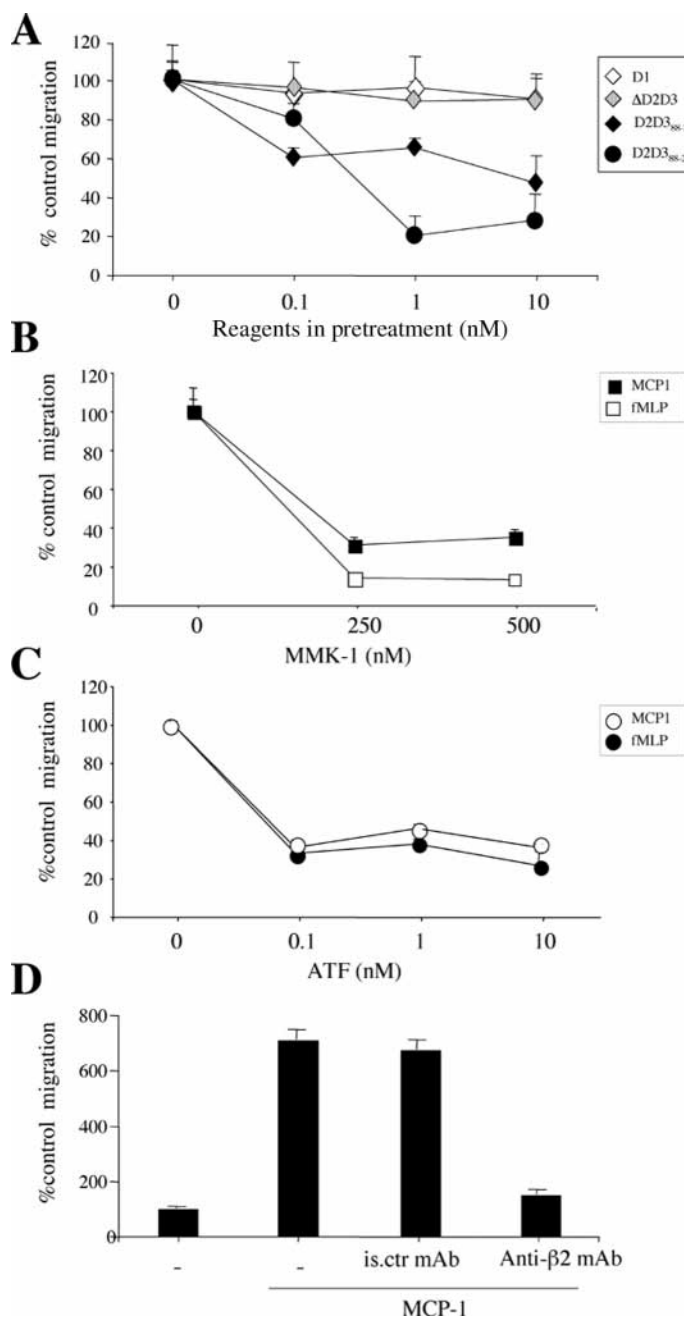
As an independent proof for receptor functionality we investigated whether D2D3$_{88-274}$ inhibits MCP-1-induced polymerization of newly formed F-actin. To this aim, the cells were either not treated or pretreated with D2D3$_{88-274}$ as described above. Thereafter, the cells were stimulated with MCP-1, fixed, permeabilized, stained with FITC-labeled phalloidin and analyzed by flow cytometry. As a control, homologous receptor desensitization was induced by pretreatment of the cells with an excess of MCP-1. The results showed a rapid and transient actin polymerization induced by MCP-1. When the cells were pretreated with MCP-1, additional MCP-1 had no effect on actin polymerization. On the contrary, pretreatment with D2D3$_{88-274}$ did not inhibit actin polymerization induced by MCP-1 (Fig. 2C). This experiment indicates that D2D3$_{88-274}$ does not prevent F-actin polymerization induced by MCP-1. Taken together, these results suggest that D2D3$_{88-274}$-dependent inhibition of MCP-1-induced monocyte migration cannot be explained by competition for MCP-1 binding, receptor down modulation or receptor desensitization.

D2D3$_{88-274}$ inhibits RANTES and fMLP-induced monocyte migration

We next investigated whether, in addition to MCP-1, D2D3$_{88-274}$ was also able to interfere with other chemoattractants, fMPL or RANTES. To this aim, monocytes (Fig. 3) and THP-1 cells (not shown) were pretreated for 15 minutes with either D1 or D2D3$_{88-274}$ at concentrations indicated in the figure. The cells were subsequently allowed to migrate in response to either fMLP or RANTES (Fig. 3A). While untreated, ΔD2D3- or D1-treated cells responded to a similar extent to fMLP; pretreatment with D2D3$_{88-274}$ compromised the ability of the cells to respond to this chemoattractant. Furthermore, D2D3$_{88-274}$ also inhibited RANTES-induced monocyte chemotaxis (Fig. 3A, closed circles). As observed for MCP-1, the inhibitory activity of D2D3$_{88-274}$ was concentration-dependent and was at its maximum at 1 nM. Indeed, fMLP- and RANTES-induced chemotaxis of D2D3$_{88-274}$-treated cells was 58% and 50%, respectively, when compared with untreated control cells.

Fig. 2. D2D3$_{88-274}$ stimulation does not induce CCR2 heterologous desensitization. (A) MCP-1 binding to CCR2 was measured on human monocytes and CCR2-overexpressing CHO cells by the addition of 1 nM ^{125}I-MCP-1 (see Materials and Methods). The percentage of control specific binding is indicated in the figure. (B) Monocytes and THP-1 cells were loaded with 1 mM Fura-2 for 30 minutes at 37°C and then stimulated with 1 μM MCP-1 or 1 μM D2D3$_{88-274}$. Fluorescence output was measured at 340 nm in a fluorescence spectrophotometer. The results were confirmed in two different experiments. (C) THP-1 cells were pretreated with serum-free medium (med), 10 nM D2D3$_{88-274}$ (D2D3) or 10 nM uPA (uPA) for 30 minutes and then stimulated with 100 nM MCP-1 for the times indicated (x-axis). At the end of the stimulation, cells were fixed, permeabilized and stained with FITC-labeled phalloidin. The amount of bound phalloidin-FITC was evaluated by flow cytometry analysis and its increase in percent, compared to the content of F-actin detected in unstimulated cells, is shown in the figure (y-axis). The data in A and C represent the mean±s.d. of three experiments. Each experiment was performed in triplicate for each stimulus.

Fig. 3. D2D3$_{88-274}$ inhibits β2-integrin-dependent monocyte migration. (A) THP-1 cells were pretreated for 15 minutes at 37°C with increasing concentrations of D1 (◇), ΔD2D3 (gray ◇), D2D3$_{88-274}$ (◆) or D2D3$_{88-274}$ (●). (B, C) THP-1 cells were pretreated for 15 minutes at 37°C with increasing concentrations of MMK-1 and ATF. After pretreatment, cells were tested for their ability to migrate in response to fMLP (A,B,C), RANTES (A) or MCP-1 (B,C). The number of cells migrating towards the different chemokines was taken as 100%, and the values of cells migrating after pretreatment with pretreating reagents were calculated relative to this. (D) THP-1 cells were incubated with either neutralizing anti-β2 mAb or with an isotype-matched control-mAb. Thereafter the cells were allowed to migrate in response to MCP-1 (2 nM). The data in A, B, C and D represent the mean±s.d. of three experiments. Each experiment was performed in triplicate for each stimulus.

D2D3$_{88-274}$ induces cell migration by interacting with FPRL-1 (Resnati et al., 2002). We next investigated whether FPRL-1 engagement by MMK-1, a synthetic ligand of FPRL-1, modulates chemokines-induced chemotaxis. To this aim, the cells were pretreated with different amounts of MMK, and then allowed to migrate in response to MCP-1 and fMLP gradients. The results reported in Fig. 3B show that MMK inhibited MCP-1- and fMLP-induced chemotaxis by 65% and 85%, respectively. Furthermore, pretreatment of the cells with either uPA (not shown) or its amino-terminal fragment (ATF) (Fig. 3C) inhibited both MCP-1- and fMLP-induced monocyte chemotaxis by 70%. Together, these results indicate that either FPRL-1 engagement by D2D3$_{88-274}$ or MMK-1, or its possible interaction with the uPA/uPAR complex inhibits chemokine-induced monocyte chemotaxis.

D2D3$_{88-274}$ inhibits chemokine-induced integrin-dependent cell adhesion

In most circumstances, a phase of loose adhesion followed by a phase of firm attachment precedes migration of cells in vivo. We reasoned that D2D3$_{88-274}$ might inhibit chemokine cell migration by modulating cell adhesion. Indeed, chemokine-induced monocyte migration through the Boyden chamber filter relied on the activity of β2 integrin, and was inhibited by pretreating the cells with an anti-β2-integrin mAb but not with an isotype-matched control-mAb (Fig. 3D).

To investigate whether D2D3$_{88-274}$ regulates β2-integrin function, we exploited a rapid adhesion assay that completely relies on integrin functionality (Liddington and Ginsberg, 2002; van Kooyk and Figdor, 2000). As expected, both MCP-1 and fMLP (at 2 μM and 50 μM, respectively) induced rapid monocyte adhesion to fibrinogen-coated fiberglass (Fig. 4A). By contrast, neither D2D3$_{88-274}$ nor MMK-1 were able to induce monocyte adhesion at any of the concentrations tested (Fig. 4A). We then investigated, whether pretreatment of the cells with D2D3$_{88-274}$ affects the rapid and firm monocyte adhesion induced by MCP-1 and fMLP. To this aim, PBMCs were pretreated with 1 μM of D2D3$_{88-274}$, D1, ΔD2D3 or full length soluble uPAR (suPAR), stimulated for 3 min with either MCP-1 or fMLP and allowed to adhere to fibrinogen coated glass slides (Fig. 4B). The results indicated that D1-, ΔD2D3- and suPAR-treated cells adhered like untreated control cells, whereas pretreatment with D2D3$_{88-274}$ inhibited up to 75% of the MCP-1-induced cell adhesion. The inhibitory activity of D2D3$_{88-274}$ was detected at the concentration of 1 μM and was rapidly lost at concentrations below 10 nM (data not shown). Interestingly, suPAR failed to inhibit MCP-1- and fMLP-induced cell adhesion at all concentrations tested (0.2 μM, 0.5 μM and 1 μM) (Fig. 4C). Similarly to D2D3$_{88-274}$, MMK-1 also inhibited MCP-1- and fMLP-induced cell adhesion (Fig. 4B). D2D3$_{88-274}$ and MMK-1 failed to inhibit MCP-1- and fMLP-induced adhesion when added simultaneously to the chemokine, confirming that receptor competition does not account for the observed inhibitory activity (control mix in Fig. 4B). To investigate whether the inhibitory activity of both D2D3$_{88-274}$ and MMK-1 required FPRL-1, we pretreated the cells with an anti-FPRL-1 mAb capable of inhibiting both D2D3$_{88-274}$- and MMK-1-induced cell migration (Resnati et al., 2002) and data not shown. Thus, monocytes were first pretreated with

the anti-FPRL-1 Ab or control Ig, then incubated with D2D3$_{88-274}$ or MMK-1, stimulated with either MCP-1 or fMLP and allowed to adhere to fibrinogen coated glass slides. Neither the anti-FPRL-1 Ab, nor the control Ig interfered with MCP-1- and fMLP-induced cell adhesion. By contrast, the anti-FPRL-1 Ab, but not the control Ig, completely prevented D2D3$_{88-274}$ and MMK-1-inhibition of MCP-1- or fMLP-induced cell adhesion (Fig. 4D).

Together these results indicate that engagement of the FPRL-1 receptor by either D2D3$_{88-274}$ or MMK-1 inhibits MCP-1- and fMLP-dependent monocyte adhesion by decreasing β2-integrin activation. To investigate which intracellular signals are responsible for this event we treated

the cells with PI3K, p38 and Hck inhibitors. These inhibitors were chosen because PI3K, p38 and Hck are not directly involved in MCP-1- and fMPL-induced cell adhesion (Ashida et al., 2001; Cambien et al., 2001), but are required for D2D3$_{88-274}$- and uPA-dependent cell migration (Resnati et al., 1996; Webb et al., 2000). PBMCs were first treated with the indicated concentration of the inhibitors, then stimulated with D2D3$_{88-274}$, and then allowed to adhere in response to either MCP-1 or fMLP (Fig. 4E). Inhibitors of PI3K, p38 or Hck did not prevent the inhibitory activity of D2D3$_{88-274}$, thus suggesting that D2D3$_{88-274}$ inhibits chemokine-induced monocyte adhesion through an intracellular pathway that does not involve these kinases.

Fig. 4. D2D3$_{88-274}$ inhibits chemokine-induced monocyte-adhesion. Rapid adhesion of monocytes to fibrinogen-coated slides was assessed as described in Materials and Methods. Adherent cells were counted in four independent 0.2 mm^2 fields by using video-imaging software. Triplicates were analyzed for each condition. The data are the mean±s.d. of triplicate evaluation (a total of twelve 0.2 mm^2 fields). (A) Monocytes were stimulated with MCP-1 (2 μM), fMLP (50 μM) or the indicated amounts of D2D3$_{88-274}$ and MMK-1 (MMK) and allowed to adhere for 3 minutes. (B) Monocytes were left untreated (–) or pretreated for 15 minutes with D2D3$_{88-274}$ (1 μM), MMK (50 μM), D1 (1 μM), ΔD2D3 (1 μM) or full-length suPAR (1 μM). The ability of the cells to adhere to fibrinogen-coated slides was determined following stimulation with MCP-1 (black bars) or fMLP (white bars). As control, D2D3$_{88-274}$ was added to the cells simultaneously with MCP-1 or fMLP stimulation (Ctr-MIX). Gray bar, basal adhesion. (C) For titration: pretreatment for 15 minutes with suPAR at concentrations as indicated. Gray bar, basal adhesion (CTR); black bars, MCP-1 (1 μM). (D) Monocytes were left untreated (–) or preincubated with either anti-FPRL-1 Ab (αFPRL-1) or control Ig (Ctr Ig) for 15 minutes on ice after which they were treated for an additional 15 minutes at 37°C with medium (–), 1 μM D2D3$_{88-274}$, or 50 μM MMK. The ability of the cells to adhere to fibrinogen-coated slides was then determined following stimulation with MCP-1 (black bars) or fMLP (white bars). Gray bar, basal adhesion. (E) Monocytes were incubated with medium (–), the Hck inhibitor PP2 (10 nM), the PI3K inhibitor LY (25 μM) and the p38 inhibitor SB (1 μM) for 30 minutes at 37°C. Thereafter, half of the cells were treated with D2D3$_{88-274}$ (1 μM) for an additional 15 minutes at 37°C. Following this, all cells were stimulated with MCP-1 (2 μM) and allowed to adhere to fibrinogen-coated slides for 3 minutes. Gray bar, basal adhesion.
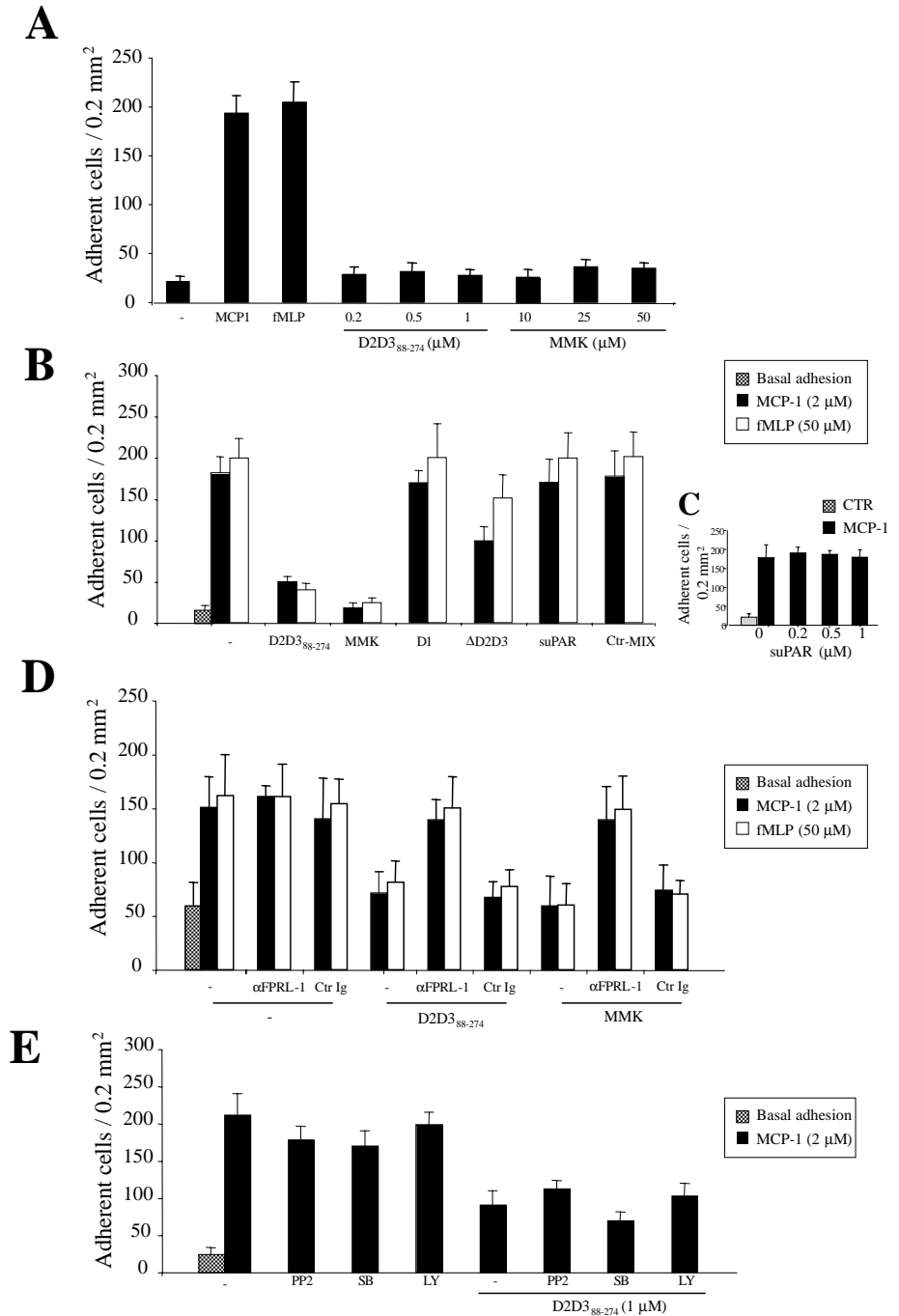
Discussion

Here we have shown that D2D3$_{88-274}$, the chemotactic fragment of soluble uPAR, inhibits chemokine-induced monocyte chemotaxis by preventing rapid integrin-dependent cell adhesion. We suggest to consider this as a novel and possibly general mechanism involved in the regulation of inflammatory responses.

Our previous findings (Blasi and Carmeliet, 2002; Fazioli et al., 1997; Resnati et al., 1996; Resnati et al., 2002) and the results reported here, indicate that upon cleavage and release from the cell surface, uPAR-derived D2D3$_{88-274}$ is endowed with all properties of a chemoattractant, i.e. it is able to both positively and negatively regulate monocyte migration. Indeed, D2D3$_{88-274}$ can induce monocyte migration and can interfere with MCP-1-, RANTES- and fMLP-dependent cell migration.

It is interesting that, like the chemotactic activity, the inhibitory activity of D2D3$_{88-274}$ also requires the amino acidic sequence in the D1-D2 linker region mapping between residues 88 and 92. However, whereas chymotrypsin-generated D2D3$_{88-274}$ and recombinant D2D3$_{84-274}$ both inhibited MCP-1-induced cell migration, neither D1 nor ΔD2D3 (which lacks the sequence SRSRY 88-92) elicited a similar effect. Consistently, full-length suPAR, previously shown to lack chemotactic activity, failed to alter chemokine-induced cell migration. Interestingly, uPA and ATF mimicked the effect of D2D3$_{88-274}$ and D2D3$_{84-274}$, and inhibited MCP-1- and fMLP-induced cell migration. These data allow the conclusion that the 88-92 fragment of uPAR, exposed upon receptor cleavage or following uPA or ATF binding, is responsible for both cell migration (Fazioli et al., 1997) and chemokine cross-regulation. Our findings also indicate that both activities are probably mediated by the engagement of FPRL-1. Thus, the D2D3$_{88-274}$ induced cell migration is inhibited by anti-FPRL-1 Ab (Resnati et al., 2002), and MMK-1, a synthetic ligand of this receptor, and recapitulates the D2D3$_{88-274}$-dependent inhibition of both MCP-1 and fMLP-induced chemotaxis (Fig. 3).

What is the mechanism responsible for D2D3$_{88-274}$- and MMK-dependent inhibition of chemokine-induced cell migration? Chemokine receptor desensitization has been previously demonstrated and generally occurs via homologous or heterologous desensitization (Ali et al., 1999). Our finding that D2D3$_{88-274}$ prevents MCP-1-, RANTES- and fMLP-mediated cell migration suggests that D2D3$_{88-274}$ mediates either heterologous (CCR2, CCR5) or homologous (FPRL-1) receptor desensitization. However, we found that D2D3$_{88-274}$ was unable to inhibit MCP-1 receptor binding, to elicit CCR2 internalization, to prevent MCP-1-mediated Ca^{2+} flux or to alter MCP-1-mediated actin polymerization. Likewise, D2D3$_{88-274}$ pretreatment did not elicit FPRL-1 downregulation and did not inhibit fMLP-mediated Ca^{2+} flux in FPRL-1-expressing cell lines (M.R. and F.B., unpublished data). Thus, CCR2 and FPRL-1 receptor desensitization cannot explain D2D3$_{88-274}$-dependent inhibition of MCP-1- and fMLP-mediated cell migration. Even though we did not specifically address whether D2D3$_{88-274}$ pretreatment induces CCR5 heterologous desensitization, this seems unlikely in light of the results above reported. Rather, we favor the possibility that the engagement of FPRL-1 by D2D3$_{88-274}$ or MMK-1, or the association of the FPRL-1 receptor with the uPA/uPAR complex originate an intracellular signal able to desensitize the cells to chemokine-induced chemotaxis. Since chemokine-directed cell migration

is preceded by a phase of integrin-dependent cell adhesion, we postulate that inhibition of this event hampers the ability of the cells to further respond to the chemokine gradient. Indeed, by taking advantage of a rapid adhesion assay, which relies on integrin activation, we found that pretreatment of monocytes with D2D3$_{88-274}$ prevented MCP-1- and fMLP-induced rapid cell adhesion. As for chemotaxis, the ability of D2D3$_{88-274}$ to inhibit integrin-dependent cell adhesion was attributable to the D1-D2D3 linker region (amino acids 88-92). Thus, while chymotrypsin-generated D2D3$_{88-274}$ and recombinant D2D3$_{84-274}$ inhibited MCP-1- and fMLP-induced cell adhesion to similar extents, D1 and ΔD2D3 failed to do so. Moreover, full-length suPAR was unable to alter chemokine-induced cell adhesion at all concentrations tested. Our data indicate that like the chemotactic activity, the inhibitory activity of D2D3$_{88-274}$ also requires the interaction with FPRL-1. Thus, the inhibitory effect of D2D3$_{88-274}$ was prevented by the addition of anti-FPRL-1 Ab, and it was recapitulated by the FPRL-1 ligand MMK. Since only full length membrane-bound uPAR (Aguirre Ghiso et al., 1999; Bohuslav et al., 1995; Montuori et al., 2002; Simon et al., 1996; Simon et al., 2000; Sitrin et al., 1996; Wei et al., 1996) but not truncated forms of uPAR (that lack the D1 domain and expose the 88-92 linker region) can bind β2 integrin and β1 integrin (May et al., 1998; Montuori et al., 2002), we suggest that D2D3$_{88-274}$ regulates integrin function by signaling via FPRL-1 rather than via direct binding to the β2 integrins.

Integrin affinity and avidity are generally regulated by inside-out signaling, which involves ligand-independent integrin clustering and high affinity states, and/or ligand-induced conformational changes. What could be the signaling pathway involved in D2D3$_{88-274}$- and MMK-dependent inhibition of β2-mediated cell adhesion? Several signaling molecules are involved in inside-out signaling leading to integrin activation by chemokines (Ashida et al., 2001; Laudanna et al., 2002). We show here that PP2, a potent and specific inhibitor of the Src family of tyrosine kinases, LY, an inhibitor of the PI3K, and SB, an inhibitor of p38, have no effect on D2D3$_{88-274}$-dependent inhibition of integrin functions. It is possible that the regulation of integrin function requires intracellular signaling events different from the ones mediating cell migration. This could be in keeping with the observation that the regulation of integrin-dependent cell adhesion requires maximal receptor engagement, rather than the gradient needed for chemotaxis. Interestingly, whereas both D2D3$_{88-274}$ and MMK-1 bind to the same receptor (FPRL-1), they elicit different intracellular signaling pathways. Indeed, whereas MMK-1 induces Ca^{2+} fluxes (Hu et al., 2001), D2D3$_{88-274}$ fails to do so on monocytic cells (Fig. 2) and FPRL-1 overexpressing cells (M.R. and F.B., unpublished data). Despite this difference they both regulate integrin function. We are currently investigating whether integrin sequestration or inhibition of chemokine-induced integrin clustering, accounts for the ability of D2D3$_{88-274}$ and MMK to regulate chemokine-induced cell adhesion.

In conclusion, our results revealed an unexpected activity for uPAR/FPRL-1 and underlined the existence of a new mechanism regulating the complex interaction between chemoattractants and integrins. Thus, inhibition of chemokine-induced integrin-dependent cell adhesion should be considered in addition to chemokine receptor desensitization, as a possible mechanism that can prevent monocytes recruitment in the

inflamed site. In this light, it is interesting that increased levels of soluble uPAR and soluble D2D3$_{88-274}$ are found in the blood of neoplastic patients. In fact, the D2D3 concentration in the serum of leukemia and cancer patients is similar to that of full-length suPAR (0.1-2 nM), i.e. the chemotactically active concentration (Sidenius and Blasi, 2003). It is tempting to speculate that tumor-released D2D3$_{88-274}$ conditions the tumor microenvironment by affecting the onset of a local inflammatory reaction that is required to mount a proper immune response against the tumor. This possibility is currently under investigation.

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