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Endocytic receptor LRP together with tPA and PAI-1 coordinates Mac-1-dependent macrophage migration

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Migration of activated macrophages is essential for resolution of acute inflammation and the initiation of adaptive immunity. Here, we show that efficient macrophage migration in inflammatory environment depends on Mac-1 recognition of a binary complex consisting of fibrin within the provisional matrix and the protease tPA (tissue-type plasminogen activator). Subsequent neutralization of tPA by its inhibitor PAI-1 enhances binding of the integrin–protease–inhibitor complex to the endocytic receptor LRP (lipoprotein receptor-related protein), triggering a switch from cell adhesion to cell detachment. Genetic inactivation of Mac-1, tPA, PAI-1 or LRP but not the protease uPA abrogates macrophage migration. The defective macrophage migration in PAI-1-deficient mice can be restored by wild-type but not by a mutant PAI-1 that does not interact with LRP. *In vitro* analysis shows that tPA promotes Mac-1-mediated adhesion, whereas PAI-1 and LRP facilitate its transition to cell retraction. Our results emphasize the importance of ordered transitions both temporally and spatially between individual steps of cell migration, and support a model where efficient migration of inflammatory macrophages depends on cooperation of three physiologically prominent systems (integrins, coagulation and fibrinolysis, and endocytosis).

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Introduction

Inflammation is associated with increased vascular permeability, activation of the coagulation pathway, and the formation of fibrin clots. In response to acute inflammation, monocytes/macrophages accumulate at the site of injury (Leibovich and Ross, 1975), where they participate initially in innate immune responses and then in wound healing. During resolution of the inflammation, they migrate in a Mac-1 ($\alpha_M\beta_2$, CD11b/CD18, CR3)-dependent manner (Cao et al, 2005) from the site of injury to the lymphatic system (Bellingan et al, 1996), where they present antigens to lymphocytes and help maintain immune surveillance. Failure of monocyte/macrophage efflux from the site of injury to the draining lymph nodes contributes in part to the pathogenesis of many chronic inflammatory diseases, including atherosclerosis (Libby, 2002; Llodra et al, 2004). Thus, understanding the mechanisms controlling macrophage migration within different environments is of paramount importance.

Cell migration is a complex process involving multiple steps, including intracellular signaling, cell adhesion, cytoskeletal reorganization, cell detachment, and recycling of cell surface receptors (Stossel, 1994; Murphy and Gavrilovic, 1999; Sanchez-Madrid and del Pozo, 1999; Webb et al, 2002), and therefore requires participation of a wide range of biological processes (Bretscher, 1984; Murphy and Gavrilovic, 1999). In particular, the low-density lipoprotein receptor-related protein (LRP), an endocytic receptor that recognizes a wide range of ligands, including protease–protease inhibitor complexes, has been shown to modulate cell migration in several *in vitro* systems (Herz and Strickland, 2001), in part via its interaction with the uPA/uPA receptor (uPAR)/integrin complex (Wei et al, 1996; Czekay et al, 2003). However, the effects of the LRP/uPA/uPAR system on cell migration *in vivo* remains to be tested, especially given the fact that no substantial migration defects have been reported for uPAR-deficient mice (Dewerchin et al, 1996).

In this work, we have studied the roles of PAI-1 and LRP in macrophage migration in response to inflammation. These results support a novel mechanism underlying the Mac-1-dependent migration of activated macrophages within an inflammatory environment, where tissue-type plasminogen activator (tPA), its specific inhibitor PAI-1, and the endocytic receptor LRP work together to facilitate the transition between the individual steps of cell migration. Specifically, we show that tPA initiates macrophage adhesion by enhancing Mac-1 recognition of fibrin, which is present in the provisional matrices and shown to play a critical role in macrophage adhesion *in vivo* (Szaba and Smiley, 2002). This is followed by PAI-1 inhibition of tPA, which in turn couples the adhesion complex to LRP, and leads to the internalization of the adhesion complex and the detachment of the cell trailing edges via LRP-mediated endocytosis. Together, these

data provide new mechanistic insight in macrophage migration during inflammation, a process that is critical to many physiological and pathological events. Significantly, our results demonstrate the importance of an ordered transition for the individual steps of cell migration, and provide novel and detailed mechanistic information that may help us better understand how the different steps of cell migration are coordinated on a molecular level.

Results

tPA is required for efficient macrophage adhesion to fibrin(ogen)

Proper host response to an acute injury requires both the initial infiltration of blood leukocytes to the site of injury and the subsequent efflux of inflammatory macrophages during resolution of inflammation (Bellingan *et al*, 2002). Although infiltration of blood neutrophils and monocytes through the endothelium is well studied, little is known about the mechanism underlying the emigration of mature macrophages to the lymphatics. Recently, Bellingan *et al* (2002) reported that unstimulated macrophages migrate spontaneously over a 4-day period to the lymph nodes in a β_1-integrin-dependent manner. We showed that the efficiency of such macrophage emigration is dramatically increased upon LPS stimulation, potentially owing to switching from a β_1 to a Mac-1-dependent process (Cao *et al*, 2005). Indeed, we found that function-blocking antibodies of VLA-4 and VLA-5 did not have significant effects on *in vivo* macrophage migration in the presence of LPS (Supplementary data).

Given that genetic inactivation of fibrin(ogen), a ligand of Mac-1 (Altieri *et al*, 1993), also leads to defective macrophage adhesion/migration *in vivo* (Szaba and Smiley, 2002), we hypothesized that macrophage migration under inflammatory conditions requires Mac-1 and its recognition of fibrin (ogen). As Mac-1 binds fibrin(ogen) with relatively low affinity (Altieri *et al*, 1993), we speculated that additional factors may be involved that promote specifically Mac-1-dependent migration. tPA, which is known to interact with fibrin at a site close to the Mac-1 recognition sequences (P1 and P2) (Altieri *et al*, 1993; Ugarova *et al*, 1998; Medved and Nieuwenhuizen, 2003), could provide this cofactor activity. To test this hypothesis, we conducted cell adhesion experiments using human 293 cells that stably express recombinant Mac-1 (Zhang and Plow, 1996), and the fibrin DDE fragment that contains two fibrinogen D domains and one central E domain (Olexa and Budzynski, 1979). The addition of tPA to immobilized DDE increased Mac-1-dependent cell adhesion in a concentration-dependent manner, which could be blocked by the addition of the specific Mac-1-antagonist neutrophil inhibitory factor (NIF) (Muchowski *et al*, 1994) (Figure 1A). Thus the tPA/fibrin complex leads to enhanced Mac-1-mediated cell adhesion.

tPA is a novel Mac-1 ligand

One of the mechanisms by which tPA may promote Mac-1-mediated adhesion to fibrin(ogen) is by direct interaction with Mac-1, thus converting a monovalent interaction between Mac-1 and either fibrin or tPA into a bivalent interaction between Mac-1 and the tPA/fibrin complex. To test this hypothesis, we conducted solid-phase binding assays in two different formats with either purified Mac-1 (Figure 1B) or
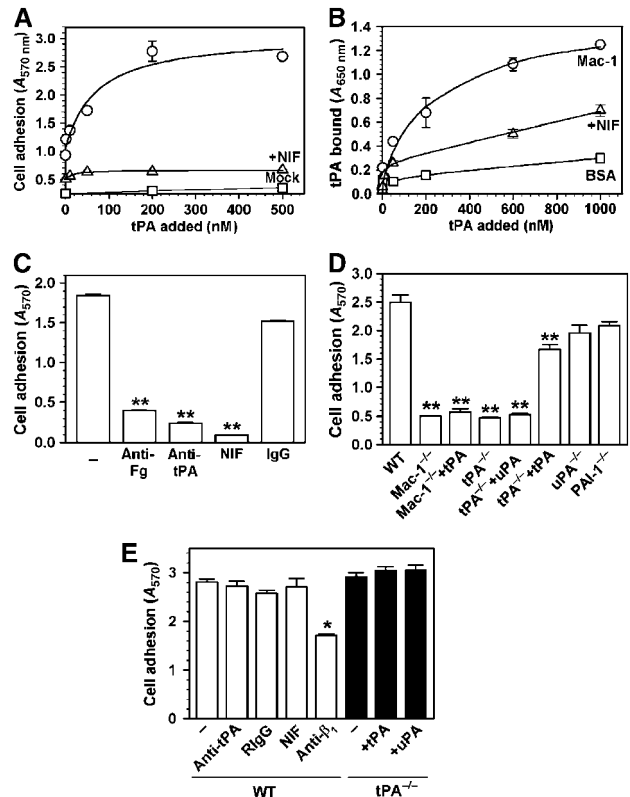

Figure 1 tPA binds Mac-1 and promotes cell adhesion to fibrin. (**A**) Mac-1-expressing (○) or mock (□) 293 cells were added to DDE (50 μg/ml)-coated 24-well plates in the presence of different concentrations of tPA without or with NIF (100 nM) (△), and the number of adherent cells was quantified. (**B**) Different concentrations of tPA were added to Mac-1-coated 96-well plates in the presence (△) or absence (○) of NIF (100 nM). The amount of bound tPA was detected using anti-tPA antibody and an HRP conjugate of secondary antibody. For both (A) and (B), the data shown are the means ± s.d. and are representative of two to three independent experiments. (**C, D**) Fresh macrophages isolated from WT (C) or different knockout mice with or without add-back of tPA (50 nM) or uPA (50 nM) (D) were allowed to adhere to DDE (50 μg/ml)-coated wells. Macrophage adhesion was measured as in (A). In panel C, WT macrophages were treated, prior to adhesion assays, with anti-Fg (50 μg/ml), anti-tPA (50 μg/ml), NIF (100 nM), or a control IgG. The data shown are the means ± s.d. (*n* = 3). *Student's *t*-test *P* < 0.005. (**E**) Fresh macrophages isolated from WT or tPA-deficient mice with or without add-back of tPA (50 nM) or uPA (50 nM), or pretreatment with anti-tPA (50 μg/ml), NIF (100 nM), anti-β_1 (9EG7, 20 μg/ml), or a control IgG, were allowed to adhere to fibronectin-coated wells (10 μg/ml). Macrophage adhesion was measured as in (A). The data shown are the means ± s.d. (*n* = 3). *Student's *t*-test, *P* < 0.05.

purified tPA (data not shown) immobilized to microtiter wells. In both cases, the reciprocal ligand bound specifically in a dose-dependent manner. In addition, NIF blocked tPA binding to Mac-1 (Figure 1B). These data indicate that purified Mac-1 binds to purified tPA. In further support, we found that Mac-1 transfected but not mock transfected human 293 cells adhered directly to tPA, and this adhesion could be blocked by NIF (data not shown). No adhesion was seen to a control protein (ovalbumin).

To see if tPA could also promote Mac-1-mediated macrophage adhesion, we conducted cell adhesion assays as above using freshly obtained peritoneal macrophages from mice. We found that, in agreement with the above results, the

Mac-1 antagonist, NIF, as well as polyclonal antibodies against fibrinogen or tPA could block cell adhesion (Figure 1C), demonstrating the contribution of these molecules in the adhesion of macrophages. Since no endogenous tPA was added to this assay, these data imply that the adhesion of wild-type (WT) macrophages utilizes endogenous tPA produced by the macrophages. Indeed, a significant amount of tPA could be detected on the cell surface of freshly purified macrophages by FACS analysis (data not shown). Most importantly, when similar cell adhesion assays were performed using Mac-1$^{-/-}$ or tPA$^{-/-}$ macrophages, these cells exhibited dramatically reduced adhesion ($\sim$5-fold less than WT; Figure 1D). Moreover, the defective adhesion of the tPA$^{-/-}$ macrophages, but not the Mac-1$^{-/-}$ cells, could be significantly restored by adding back exogenous tPA, whereas add-back of the related plasminogen activator uPA to the tPA$^{-/-}$ macrophages failed to restore cell adhesion (Figure 1D). To further examine the specificity of Mac-1 and tPA in this system, we also analyzed cell adhesion using macrophages isolated from mice deficient in uPA$^{-/-}$ or mice deficient in the tPA inhibitor PAI-1 (PAI-1$^{-/-}$) as well as macrophage adhesion to another extracellular matrix protein fibronectin. These data demonstrated that cell adhesion to fibrin was not affected by either uPA or PAI-1 deficiency (Figure 1D), suggesting that macrophage adhesion to fibrin requires the integrin Mac-1 and its specific recognition of tPA, but is independent of either uPA or PAI-1. In contrast, cell adhesion to fibronectin is not dependent on Mac-1 or tPA, since addition of the Mac-1-specific antagonist NIF, or blocking antibodies to tPA, or genetic inactivation of tPA had no effect on macrophage adhesion to fibronectin (Figure 1E).

tPA and PAI-1 are required for Mac-1-dependent macrophage migration

Given the critical role of tPA in Mac-1-dependent cell adhesion to fibrin (Figure 1), we analyzed whether tPA also plays a role in macrophage migration. To test this, *in vitro* migration assays, using the Boyden chamber-type transwells, were performed on macrophages isolated from the tPA$^{-/-}$ mice. These data indicated that genetic inactivation of tPA results in a 18-fold reduction in macrophage migration on fibrin (Figure 2A) but not on fibronectin (Figure 2B). In contrast, migration was essentially normal with macrophages from both uPA$^{-/-}$ mice and mice deficient in the uPAR (uPAR$^{-/-}$) (Figure 2A). However, when macrophages deficient in the tPA inhibitor PAI-1 (PAI-1$^{-/-}$) were analyzed, migration was reduced by 4.8-fold. Thus, Mac-1, tPA and fibrin are important for both macrophage adhesion and migration *in vitro*, whereas PAI-1 is not required for macrophage adhesion (Figure 1D), but is critical for cell migration on fibrin. To confirm that tPA and PAI-1 are also important for macrophage migration *in vivo*, we evaluated macrophage efflux out of the peritoneum, using the same four gene-knockout mice (tPA$^{-/-}$, uPA$^{-/-}$, uPAR$^{-/-}$, and PAI-1$^{-/-}$). This *in vivo* model of macrophage migration has been described and characterized by us (Cao *et al*, 2005) and others (Szaba and Smiley, 2002), where both we and Szaba *et al* showed that in response to LPS stimulation, mature macrophages migrate across the peritoneal mesothelium to the lymphatics in both a Mac-1- and fibrin-dependent manner. Thus, macrophage migration can be measured by the difference in the total lavaged leukocyte number between the LPS-treated mice and
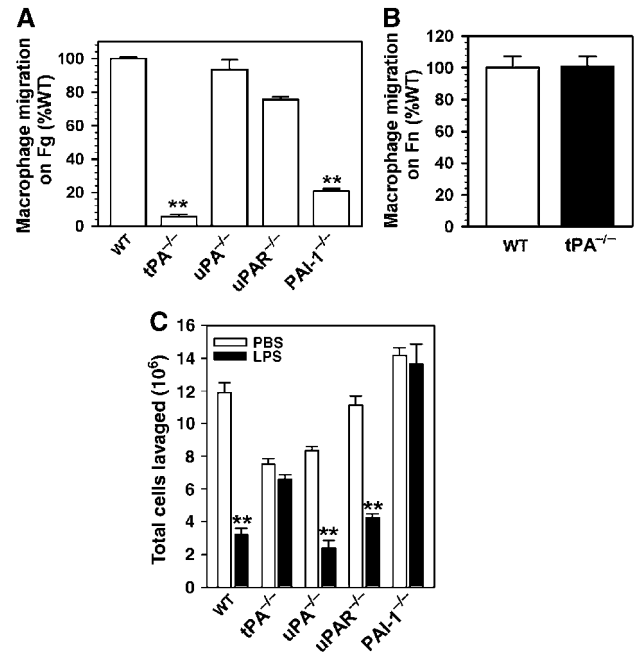

Figure 2 Macrophage migration *in vitro* and *in vivo* depends on tPA and PAI-1 but not on uPA or uPAR. (**A, B**) Macrophages obtained from different deficient mice were subjected to *in vitro* migration assays using fibrin(ogen)- (10 μg/ml) (A) or fibronectin-coated transwell plates (10 μg/ml) (B). The number of WT macrophages that migrated into the lower chamber in 4 h at 37°C was set at 100%. The data shown are the means±s.d. ($n=3$). (**C**) Macrophage migration to the draining LNs was measured *in vivo* by the difference in the number of peritoneal leukocytes between the LPS-treated (filled bars) animals and the PBS-treated (open bars) controls. The data shown are the means±s.d. ($n=6$). **Student's *t*-test, $P<0.003$.

the control PBS-treated mice. In this assay, the infiltrated monocytes, induced by intraperitoneal (i.p.) thioglycollate (TG) injection, were allowed to mature into macrophages over 4 days. Macrophage migration was then initiated by i.p. injection of LPS, and 4 h later, the total peritoneal cells were retrieved by lavage. We found that as *in vitro*, both tPA$^{-/-}$ and PAI-1$^{-/-}$ mice exhibited defective macrophage migration *in vivo*, and no significant difference in the peritoneal cell numbers was observed between the LPS- (Figure 2C, filled bars) and PBS-treated mice (Figure 2C, open bars), indicating that the majority of the PAI-1$^{-/-}$ and tPA$^{-/-}$ macrophages (95 and 93% respectively) remained within the peritoneal cavity. In contrast, both uPA$^{-/-}$ and uPAR$^{-/-}$ mice had normal macrophage migration in response to LPS stimulation, resulting in similar reductions in the peritoneal cell numbers between the LPS-treated mice (Figure 2C, filled bars) and PBS-treated mice (Figure 2C, open bars) (WT, 75%; uPA$^{-/-}$, 70%; and uPAR$^{-/-}$, 64%). Moreover, the LPS-stimulated loss of macrophages could be inhibited by the Mac-1 antagonist NIF (Cao *et al*, 2005) but not by blocking antibodies specific for integrin α_4 or α_5 (Supplementary data). These data indicate that like Mac-1 (Cao *et al*, 2005) and fibrin (Szaba and Smiley, 2002), tPA and PAI-1 are also critical for macrophage migration *in vitro*.

A critical role of LRP in macrophage migration

The above data suggest that Mac-1, fibrin and tPA form an adhesive complex, whereas PAI-1 likely functions at a different step during cell migration on fibrin. One possibility

is that inhibition of tPA by PAI-1 could promote the removal of tPA from the fibrin matrix and thereby destabilize the Mac-1/tPA/fibrin adhesion complex. This scenario is suggested by previous studies that have shown that upon interaction with a target protease, such as tPA, PAI-1 undergoes a conformational change that leads to high-affinity binding to LRP (Stefansson et al, 1998). Therefore, to determine if LRP plays a role in macrophage migration, we conducted in vitro cell adhesion and migration assays with and without the LRP antagonist the receptor-associated protein (RAP), a 39 kDa protein that inhibits all known LDLR family proteins including LRP (Strickland et al, 1990). We found that addition of either RAP or function-blocking antibodies against LRP dramatically inhibited in vitro macrophage migration (Figure 3A), but not cell adhesion (data not shown), and that i.p. injections of RAP significantly blocked the loss of

macrophages from the peritoneal cavity (Figure 3B). As PAI-1 and LRP are required for migration, but have no effect on adhesion, these data suggest that PAI-1 and LRP are not part of the initial adhesion complex and instead they act at a different step during macrophage migration on fibrin, most likely during the transition between cell adhesion and detachment.

Cre/Lox-mediated genetic inactivation of LRP inhibits macrophage migration

To confirm the importance of LRP in macrophage migration genetically, we performed tissue-specific inactivation of the LRP gene in macrophages using a Cre/Lox system. This was necessary since the complete deletion of LRP is embryonic lethal (Herz et al, 1992). The inactivation of LRP was carried out in macrophages isolated by peritoneal lavage following TG injection from homozygous mice carrying a floxed LRP gene (Rohlmann et al, 1996). These isolated macrophages were infected with a recombinant adenovirus carrying the Cre gene and a marker gene (GFP). Expression of Cre in macrophages from the LRP-floxed mice, but not from control WT mice, resulted in the loss of LRP surface expression (Figure 3C), and importantly, in a significant reduction in cell migration in vitro (Figure 3D). To exclude potential influences of viral infection on macrophage migration, we also infected macrophages from both WT mice and the LRP-floxed mice with a control adenovirus that carries only the GFP gene. These cells demonstrated both normal LRP expression (Figure 3C) and macrophage migration (Figure 3D).

LRP is required for efficient Mac-1 internalization

As LRP is known to cycle efficiently between the cell surface and intracellular compartments (Herz et al, 1992), LRP could promote macrophage migration by enhancing Mac-1 internalization and recycling back to the cell surface. To test this hypothesis, we measured Mac-1 internalization in the presence or absence of the LRP antagonist RAP. As shown in Figure 4A, approximately 30% of surface-labeled Mac-1 was internalized after 30 min at 37°C, and this internalization required stimulation by LPS (Figure 4A). However, in the presence of RAP (Figure 4A) or when LRP was genetically deleted (Supplementary data), the LPS-stimulated internalization was completely blocked, suggesting that LRP is required for efficient Mac-1 internalization in macrophages. Genetic inactivation of PAI-1 also abolished Mac-1 internalization, which could be restored by the addition of WT PAI-1 but not the mutant PAI-1 R^{76}E that inhibits tPA normally but binds LRP with markedly reduced affinity (Stefansson et al, 1998) (Figure 4B). Finally, addition of RAP did not have a significant effect on the internalization of an irrelevant receptor MHC II that is also expressed on the macrophage surface (see Supplementary data). Together, these data demonstrate that LPS-stimulated internalization of Mac-1 depends specifically on PAI-1 and LRP.

LRP colocalizes with Mac-1 on the cell surface

To determine if LRP promotes integrin internalization by colocalizing with Mac-1 on the macrophage surface, we measured the molecular proximity between these two proteins, using fluorescence lifetime imaging microscopy (FLIM) (von Arnim et al, 2004). This high-resolution imaging technique is based on fluorescence resonance energy transfer that
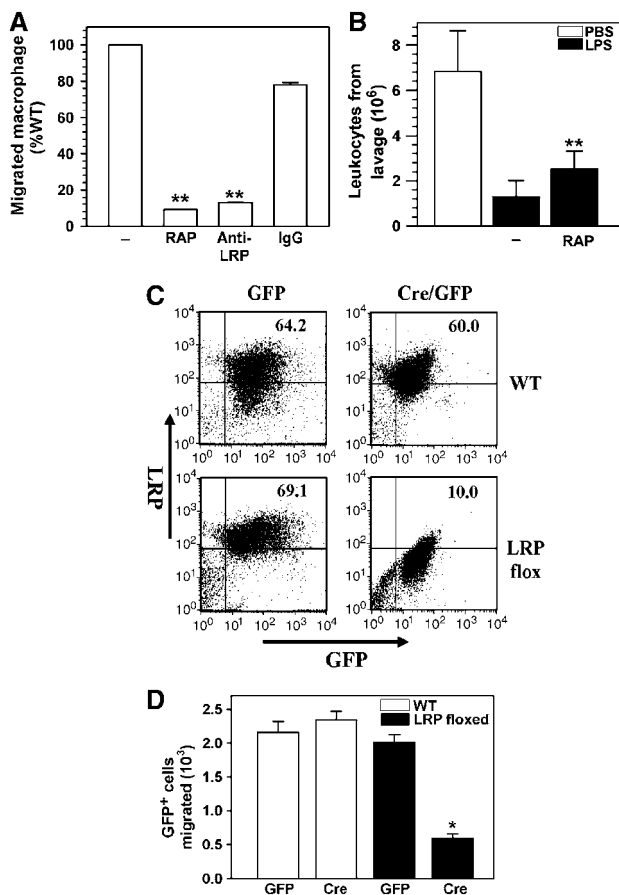

Figure 3 LRP is critical to Mac-1-dependent macrophage migration. (A) Addition of RAP (1 μM) or a LRP-specific antibody R2629 (100 μg/ml) abolished macrophage migration in vitro. The data represent the means±s.d. of three independent experiments. **Student's t-test, P<0.001, n=3. (B) I.p. administration of RAP (1 μM) blocked LPS-stimulated macrophage efflux from the peritoneum in vivo. The data represent the means±s.d. of seven mice per group. **'LPS' versus 'LPS+RAP', Student's t-test, P=0.005. (C, D) Macrophages from WT and the LRP-floxed mice were infected with adenovirus expressing GFP alone or GFP and Cre. Expression of the Cre gene but not a GFP control in the LRP-floxed macrophages abolished LRP surface expression as determined by two-color FACS analysis (C), and inhibited cell migration in vitro (D). The data represent the means±s.d. and are representative of two independent experiments. *GFP versus Cre; Student's t-test, P<0.01, n=3.
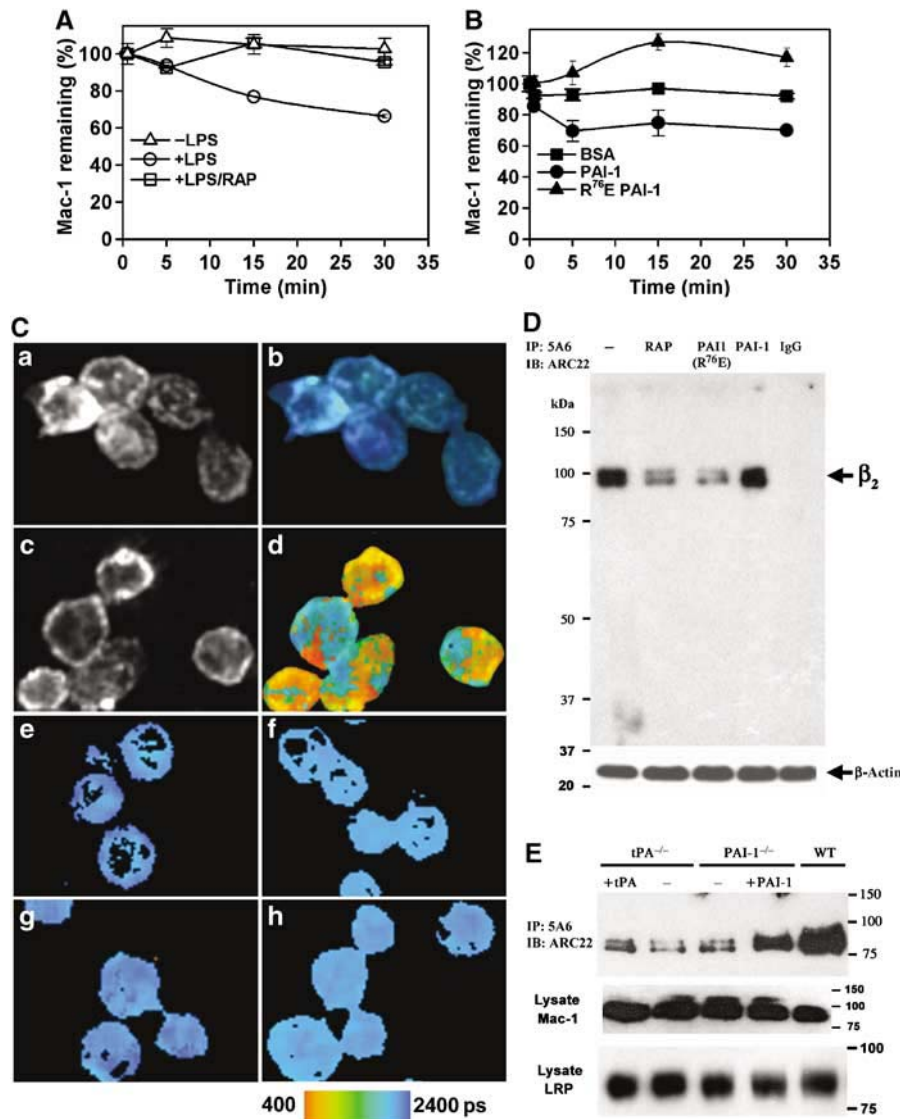
Figure 4 LRP modulates cell migration by interacting with Mac-1. (**A, B**) Surface Mac-1 on WT (A) or PAI-1$^{-/-}$ (B) macrophages was labeled with anti-Mac-1 (M1/70) at 4°C with (□) or without (○) RAP (in panel A) and with PAI-1 (●), PAI-1 (R^{76}E) (▲), or a control protein BSA (■) (in panel B). Mac-1 internalization upon incubation of macrophages in the presence (except for -Δ-) of LPS (10 ng/ml) at 37°C was measured by FACS analysis using an FITC conjugate of anti-rat IgG. The mean fluorescence intensity at time zero was assigned 100%. The data shown represent the means ± s.d. of a duplicate experiment. (**C**) Macrophages were stained with only the donor antibody (anti-LRP) (a, b), or a pair of donor and acceptor antibodies specific for LRP (R2629) and Mac-1 (M1/70) (c, d), LRP (R2629) and control rat IgG (e), control rabbit IgG and Mac-1 (M1/70) (f), MHC II (2G9) and Mac-1 (M1/70) (g), and the LRP intracellular domain (11H4) and Mac-1 (M1/70) (h), followed with Alexa 488–antidonor (rabbit or mouse) IgG and Alexa 568–anti-rat IgG. The fluorescence images (a, c) show surface staining for LRP. The FLIM images (b, d, e–h), taken at the z-section of the cell surface and presented in pseudocolors from red to blue, show the fluorescence lifetimes (in picoseconds) of Alexa 488 in the absence (b) or presence (d, e–h) of the acceptor Alexa 568. The datum shown is representative of two independent experiments. (**D, E**) Co-IP was performed with anti-LRP (5A6) or a control IgG using cell lysates from WT macrophages with or without RAP, WT PAI-1, or a mutant PAI-1(R^{76}E) (D), and using the cell lysates from either tPA$^{-/-}$ or PAI-1$^{-/-}$ macrophages with or without add-back of exogenous tPA or PAI-1, respectively (E). The IPs were separated on 10% SDS–PAGE and probed with anti-Mac-1 (ARC22) by immunoblot. Equal loading was verified by immunostaining of either total β-actin (D) or Mac-1 and LRP (E) in the lysates. The figures shown are representative of two to three independent co-IP experiments.

occurs if two fluorophores are in very close proximity (<100 Å). Accordingly, we labeled primary macrophages simultaneously with different antibody pairs, including rabbit anti-LRP extracellular domain (R2629)/rat anti-Mac-1 (M1/70), or as a control, mouse anti-LRP intracellular domain (11H4)/M1/70. Other control reactions included R2629/rat IgG, M1/70/rabbit IgG and M1/70/anti-MHC II, followed by staining with Alexa 488 and Alexa 568 conjugates of their corresponding secondary antibodies, and the fluorescence

lifetimes were determined using a multiphoton confocal microscope. We found that the presence of Mac-1 (i.e. the acceptor Alexa 568) significantly shortened the average fluorescence lifetime of the anti-LRP extracellular domain fluorophore (i.e. the donor Alexa 488) ($P < 0.001$, $n = 25$; Table I), indicating that Mac-1 and LRP resided <100 Å from each other on the cell surface. No FLIM was observed between other antibody pairs (Table I), thus confirming the specificity of colocalization between LRP and Mac-1. Figure 4C shows

Table I Association between LRP and Mac-1 on macrophages

Donor (Alexa 488)	Acceptor (Alexa 568)	Lifetime (ave. ± s.d.) (ps)	No. of cells
LRP	—	2147 ± 50	26
LRP	Mac-1	1743 ± 163*	25
LRP	IgG	2098 ± 100	24
LRP (CT)	Mac-1	2082 ± 45	26
MHC II	Mac-1	2341 ± 205	14
IgG	Mac-1	2159 ± 47	26

The fluorescence lifetimes of the donor fluorophore were calculated within each pixel that interacts with an acceptor fluorophore. A strong FLIM was observed between LRP and Mac-1, indicating that they interact with each other on the cell surface. *Student t-test, $P < 0.001$, Mac-1 versus '—' or IgG.

a representative pseudocolor-coded images of surface co-localization between LRP and Mac-1 (i.e. the z-section is set at the height of the cell surface), where a uniformly bluish image (thus long fluorescence lifetime) was obtained in the absence of the acceptor (panel b) or in the presence of a nonimmune control IgG (panel e), and a red/orange-filled image representing short fluorescence lifetime (therefore close proximity) was obtained in the presence of the acceptor Mac-1 fluorophore (panel d). Similarly, uniformly bluish images were also obtained when either a nonimmune control IgG (panel f) or MHC II (panel g) was used as the donor and Mac-1 as the acceptor. Finally, to exclude the possibility that the colocalization observed in the above experiments occurs between extracellular Mac-1 and intracellular LRP (e.g. within the endosomes), the cell surface Mac-1 was labeled with M1/70 and the intracellular domain of LRP was labeled with mAb 11H4 after cell permeabilization. A bluish image was obtained (panel h) with the 11H4/M1/70-labeled cells, demonstrating that no FLIM exists between the two receptors that reside on the opposite sides of the plasma membrane. Altogether, these experiments demonstrate that LRP associates with Mac-1 on the cell surface, and furthermore, taken with the data shown in Figure 2, they suggest that this interaction may be at least partially tPA- and PAI-1-dependent.

LRP associates with Mac-1 directly: co-immunoprecipitation (co-IP) experiments

We next examined whether LRP directly associates with Mac-1. To test this, we conducted reciprocal co-IP experiments using mAbs against LRP or Mac-1 for immunoprecipitation (IP) and antibodies against the 95 kDa β-subunit of Mac-1 or the 85 kDa subunit of LRP for immunoblots. These data indicated that Mac-1 is precipitated as a complex with LRP, and that this association is partially blocked with RAP (Figure 4D). Reciprocally, LRP could be precipitated with a mAb (M1/70) specific for Mac-1 but not by a control IgG, confirming specificity (data not shown). Moreover, the interaction of LRP with Mac-1 was inhibited by a mutant of PAI-1 (R^{76}E), which inhibits tPA normally but binds LRP with markedly reduced affinity (Stefansson et al, 1998) (Figure 4D), suggesting that the R^{76}E PAI-1 functions as a competitive inhibitor of endogenous PAI-1. To confirm that endogenous PAI-1 enhances the association of Mac-1 with LRP, we conducted additional co-IP experiments using macrophages obtained from either PAI-1$^{-/-}$ or tPA$^{-/-}$ mice. In the

absence of PAI-1, only small amounts of the LRP/Mac-1 complex could be detected, despite similar amounts of Mac-1 (Figure 4E, middle panel) and LRP (Figure 4E, bottom panel) were present in the cell lysates. Adding back exogenous PAI-1 into their corresponding cell lysates significantly enhanced the complex formation (Figure 4E). Likewise, cells that lack tPA also show reduced LRP/Mac-1 complexes and complex formation in these lysates was stimulated by the addition of tPA. Together, these data suggest that LRP associates weakly with Mac-1, a finding consistent with a recent report (Spijkers et al, 2005). However, tPA and PAI-1, both of which could be detected on the macrophage cell surface by FACS analysis (data not shown), enhance the association of LRP with Mac-1 as evidenced by co-IP analysis.

The role of PAI-1 in macrophage migration in vitro and in vivo

Based on all of the results above, we hypothesized that efficient macrophage migration depends on ordered transition among the three individual steps of macrophage migration: adhesion, movement, and integrin disengagement/internalization (Stossel, 1994; Murphy and Gavrilovic, 1999; Sanchez-Madrid and del Pozo, 1999; Webb et al, 2002). In our model, macrophage adhesion and movement requires Mac-1 recognition of the tPA/fibrin complex, whereas integrin internalization occurs when PAI-1 binds tPA and thereby couples the adhesion complex to LRP resulting in a switch from cell adhesion to receptor internalization. To test this model, we attempted to reconstitute the migratory function of the PAI-1$^{-/-}$ macrophages in vitro and in vivo, using WT PAI-1 and PAI-1 (R^{76}E) which is not recognized by LRP. For the in vitro reconstitution experiments, different concentrations of PAI-1 were mixed with the PAI-1$^{-/-}$ macrophages prior to the cell migration experiments and these results are shown in Figure 5A and B. The addition of WT PAI-1 restored the migration of the PAI-1$^{-/-}$ macrophages in a dose-dependent manner and full restoration (92% of that of WT macrophages) was achieved with PAI-1 concentrations between 1 and 10 nM. In comparison, the PAI-1 mutant (R^{76}E) failed to restore the migratory function of the PAI-1$^{-/-}$ macrophages, indicating that PAI-1 binding to LRP is critical to its ability to enhance Mac-1-mediated macrophage migration on fibrin.

Similar reconstitution experiments were also performed in vivo, by administering different concentrations of WT PAI-1 or mutant PAI-1(R^{76}E) into the peritoneum of PAI-1$^{-/-}$ mice, and measuring macrophage migration out of the peritoneum. These data demonstrated that the effect of exogenous PAI-1 on macrophage migration in vivo mirrored that of macrophage migration in vitro (Figure 5C), where efflux of the PAI-1$^{-/-}$ macrophages was increased with increasing concentrations of administered PAI-1, as evidenced by the gradual reduction of the macrophage numbers from peritoneal lavage. The optimal concentration of PAI-1 needed to fully restore the migratory function of the PAI-1$^{-/-}$ macrophages in vivo was significantly higher than the concentration required in vitro. However, in vitro the PAI-1 concentration represented the final concentration in the system, whereas in vivo there is a significant dilution of PAI-1 and the final local concentration is not known. Finally, like the reconstitution experiments in vitro, administration of the mutant PAI-1(R^{76}E) failed to restore macrophage migration

in vivo at concentrations where WT PAI-1 was fully effective (0–1 μM) (Figure 5D).

Morphological analysis of macrophage migration

The molecular and functional data presented above suggest that tPA, PAI-1 and LRP interact at different points both temporally and spatially during Mac-1-dependent macrophage migration on fibrin. To examine this possibility, we performed live cell imaging experiments on freshly isolated macrophages obtained from WT mice, treated with or without RAP, and on macrophages that were deficient in Mac-1, tPA, PAI-1, or LRP. Time-lapse images were captured at 20-s intervals and representative images are shown in Figure 6A as individual frames (video clips are available in Supplementary data). During migration the macrophages go through repeated cycles of round-polarized-round morphological changes, corresponding to the extension of the lamellipodia on the leading edge and retraction of their trailing edge. At a given time point, approximately 30–60% of the WT

macrophages were motile, and within 20 min, a majority of the migrating cells had successful retraction of their trailing extensions. Compared to WT cells, addition of RAP, or deficiency in Mac-1, PAI-1, tPA, or LRP significantly reduced the speed of cell migration ($P < 0.001$; Supplementary data). Two distinct macrophage phenotypes were observed either in the presence of RAP or from mice with gene deletions (Figure 6A). WT macrophages in the presence of RAP or macrophages with a genetic deletion of PAI-1 or LRP could extend their lamellipodia but failed to efficiently retract the trailing edge (average tail length 2.45 ± 0.2 μm for WT versus 9.4 ± 1.1 μm for WT with RAP, 9.9 ± 0.8 μm for PAI-1$^{-/-}$ and 15.2 ± 1.8 μm for LRP$^{-/-}$ cells, $P < 0.001$; Supplementary data). This suggests that interfering with LRP function or removing PAI-1 both lead to a reduction in macrophage detachment. In contrast, the Mac-1$^{-/-}$ or tPA$^{-/-}$ macrophages were able to form lamellipodia extensions, but they appeared to form weak attachments and often failed to pull the cell body forward, consistent with a role in cell adhesion.
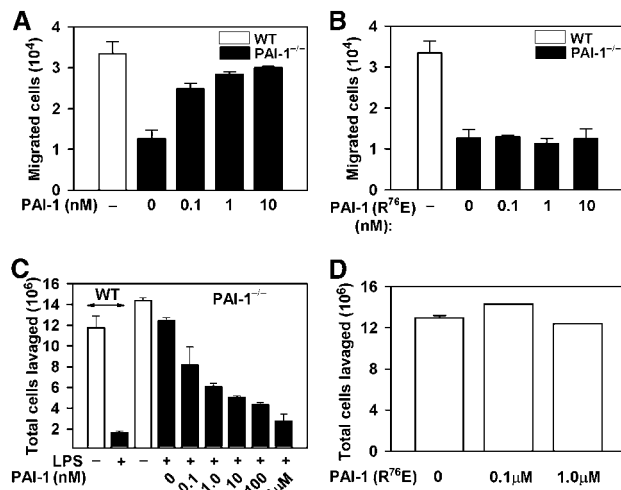
### Colocalization of LRP and Mac-1 at the trailing edges of migrating macrophages depends on tPA and PAI-1

The delayed retraction in the RAP-treated and PAI-1$^{-/-}$ cells suggests that cell detachment might be facilitated by interactions between Mac-1 and LRP at the trailing edges of the migrating macrophages. Therefore, additional colocalization studies, focusing primarily on polarized macrophages were performed. Confocal fluorescence images of migrating macrophages from the WT mice showed strong colocalization between LRP and Mac-1 (Figure 6B). Quantification (based on 120 randomly selected cells) by the algorithm using Volocity software indicated that $66.5 \pm 0.1\%$ LRP and 32.4% of Mac-1 are colocalized with each other, which agrees well with the FLIM experiment (Figure 4C). Prominent colocalization of LRP (in green) and Mac-1 (in red) was found preferentially at the cell trailing edges (Figure 6C). Furthermore, addition of RAP or genetic inactivation of PAI-1 or tPA significantly reduced LRP/Mac-1 colocalization in the trailing edges ($31.0 \pm 0.9\%$ for WT versus $4.3 \pm 0.2\%$ with RAP, $1.2 \pm 0.01\%$ for PAI-1$^{-/-}$ cells, and $5.0 \pm 0.2\%$ for tPA$^{-/-}$ cells) (Figure 6C). Add-back of exogenous PAI-1 to PAI-1$^{-/-}$ cells could partially restore LRP staining in the trailing edge and its colocalization with Mac-1 (data not shown). Mac-1 deficiency did not affect LRP distribution, and *vice versa*. Together, these results strongly suggest that colocalization between LRP and Mac-1 is required for efficient retraction of the trailing edge, thus supporting a model of macrophage migration where Mac-1 and tPA are required for firm adhesion, and PAI-1 and LRP are necessary for efficient cell retraction.

Figure 5 Reconstitution of defective macrophage migration *in vitro* and *in vivo*. (**A, B**) The PAI-1$^{-/-}$ macrophages, mixed with different concentrations of WT PAI-1 (A) or a mutant PAI-1 (R^{76}E) (B), were subjected to *in vitro* macrophage migration assays using transwell plates. Migration of the WT macrophages was determined in parallel to gauge the efficiency of reconstitution. The values shown are the means ± s.d. of a duplicate experiment and are representative of two independent experiments. (**C, D**) To rescue the defective migration phenotype of the PAI-1$^{-/-}$ mice, different concentrations of WT PAI-1 (C) or a mutant PAI-1 (R^{76}E) (D) were administered into the peritoneum of the PAI-1$^{-/-}$ mice, prior to LPS injections. The degree of restoration was determined by comparison of the PAI-1$^{-/-}$ mice to the WT mice that were treated in a similar manner. Restoration of macrophage migration was indicated by the reduced cell number from peritoneal lavage. The values shown represent the means ± s.d. of three to six mice and are representative of two independent experiments.

Figure 6 Coordination between LRP and Mac-1 is critical to macrophage migration. (**A**) Time-lapse images of macrophage migration were taken in 20-s intervals, and are presented here as individual frames of representative images (video clips were available as Supplementary data). Compared to WT macrophages, the PAI-1$^{-/-}$, LRP-deficient (LRPflox/Cre), or RAP-treated WT cells had normal lamellipodia extensions but defective retraction, and the tPA$^{-/-}$ and Mac-1$^{-/-}$ cells formed weak lamellipodia extensions. Images shown were representatives of 20 random fields per genotype taken from three independent experiments. (**B, C**) Colocalization between Mac-1 and LRP on the surface of macrophages from WT and deficient mice. Macrophages migrating on fibrin(ogen)-coated coverslips were fixed and then stained with rabbit anti-LRP (R2629) and rat anti-Mac-1 (M1/70) antibodies, followed by Alexa 488–anti-rabbit IgG and Alexa 568–anti-rat IgG. Specificity was verified using nonimmune IgGs (data not shown). The two-dimensional images (*x*–*y*) with a 0.4 μm *z*-section were taken at ×60 objective lens (B). Representative images of polarized macrophages were taken with a ×100 lens (C), showing strong colocalization at the trailing edge for WT cells, but not for PAI-1$^{-/-}$- or tPA$^{-/-}$-deficient cells or WT cells in the presence of RAP. Scale bars, 10 μm.

Discussion

Despite the extensive studies in the past two decades and the development of a generic cell migration model, composed of cell adhesion, detachment, and receptor recycling (Stossel, 1994; Murphy and Gavrilovic, 1999; Sanchez-Madrid and del Pozo, 1999; Webb *et al*, 2002), the extracellular events that orchestrate temporally and spatially the transition among
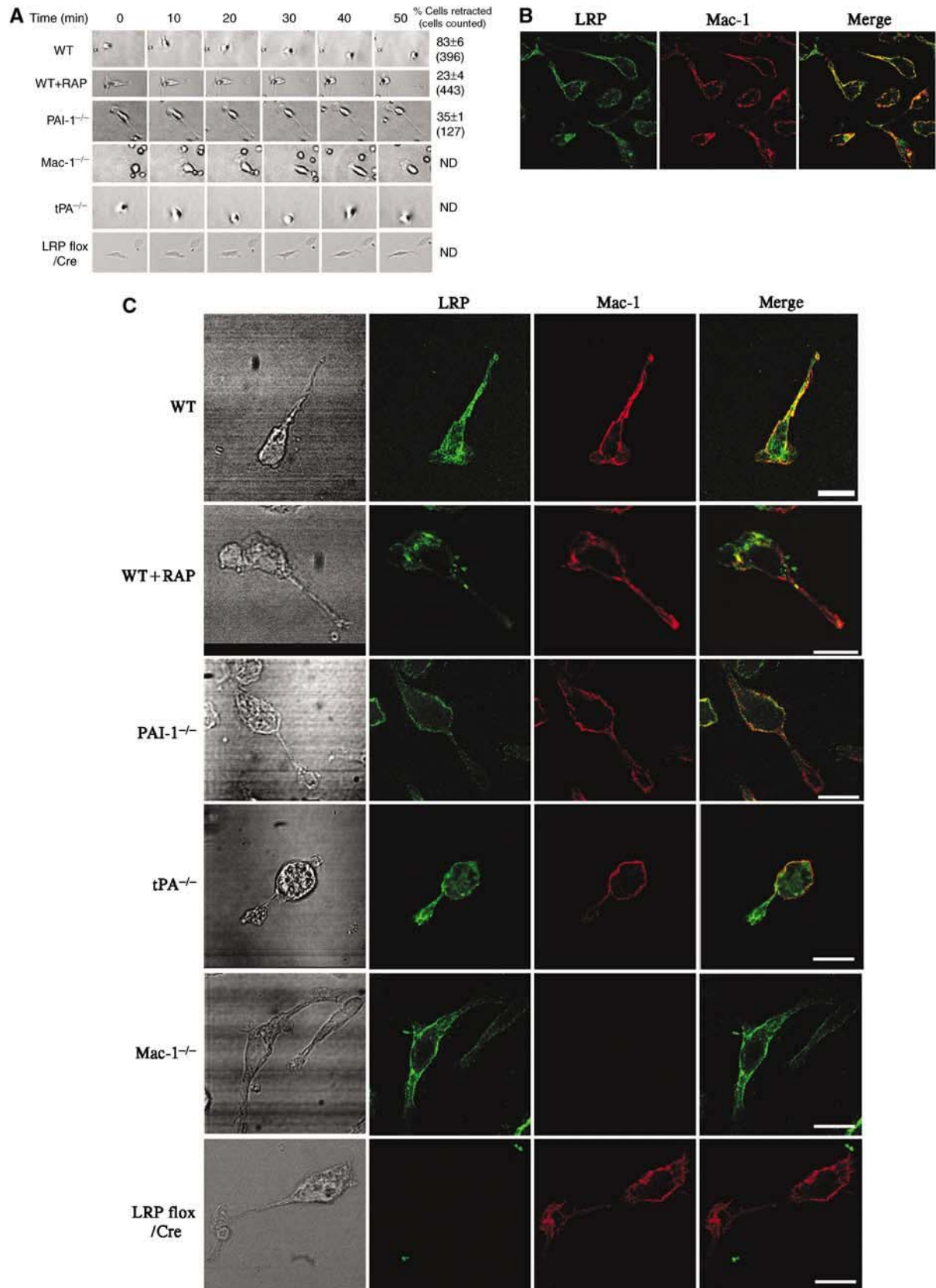
these individual steps are unknown. Moreover, evidence for a critical role of macrophage emigration to the lymph nodes in inhibiting the progression of atherosclerosis is emerging (Libby, 2002; Llodra et al, 2004). However, the mechanism that controls their emigration, especially under activated conditions, remains elusive. In this work, we explored the mechanism by which activated macrophages migrate within an inflammatory environment using genetic and biochemical approaches. Our data reveal that deletion of the PAI-1, tPA, LRP, and Mac-1 genes all impair the ability of macrophages to migrate from the peritoneal cavity in response to LPS, and to migrate in transwell assays on a fibrin matrix. Biochemical studies show that LRP associates with Mac-1 and that this association is enhanced in the presence of tPA:PAI-1 complexes. We discovered that like LRP, Mac-1 also has the ability to bind directly tPA. Together, our data are consistent with a model in which the tPA:PAI-1 complex bridges LRP with the integrin Mac-1, which in turn promotes the internalization of Mac-1 and facilitates cell detachment.

The unique feature of tPA as a Mac-1 ligand in promoting macrophage migration is its ability to link cell adhesion directly to proteolysis of fibrin, a provisional matrix that was shown by (Szaba and Smiley, 2002) to support macrophage adhesion/migration in vivo, and its ability to facilitate Mac-1 internalization by potentially linking the tPA/fibrin/Mac-1 complex to the PAI-1/LRP pathway. Therefore, the sequential interaction between Mac-1, fibrin, tPA, and PAI-1 enables the transitions among the individual steps within the cell migratory process, including cell adhesion, detachment, and receptor recycling, to occur in an ordered manner: tPA binding to fibrin and its subsequent neutralization by PAI-1 control the transitions in time; the association between LRP

and Mac-1 on the cell surface (Figure 6) controls the transitions in space; and finally, tPA/PAI-1 binding to LRP links the transitions in space and time into a single functional unit. In this regard, another plasminogen activator uPA, which also interacts with both Mac-1 and PAI-1 (Pluskota et al, 2004), and is able to promote the internalization of integrins and cell detachments via the uPA/uPAR/integrin pathway (Czekay et al, 2003), was unable to compensate for tPA in supporting macrophage migration in the tPA$^{-/-}$ mice, possibly owing to its inability to interact specifically with fibrin. Similarly, other cell surface receptors for tPA, for example, Annexin II (Ling et al, 2004), are unable to compensate Mac-1 in promoting macrophage migration in Mac-1$^{-/-}$ mice, owing to their inability to interact with PAI-1 and LRP.

Together, our data suggest a hypothesis where macrophage migration within an inflammatory environment requires well-coordinated events between thrombosis (Szaba and Smiley, 2002) and the tPA-mediated fibrinolysis (Medved and Nieuwenhuizen, 2003), as well as integrin engagement and its subsequent internalization. Specifically, we hypothesize that thrombosis, which is often associated with inflammation, generates the necessary fibrin-rich provisional matrix; the subsequent formation of the tPA/fibrin complex (Medved and Nieuwenhuizen, 2003) promotes macrophage adhesion via the integrin Mac-1 and also ensures that the adhesion complex is transient and can be removed as cells move forward. Cell detachment is achieved by tPA-mediated degradation of the fibrin polymer and by linking the Mac-1/fibrin/tPA adhesion complex to the endocytic receptor LRP via PAI-1, which triggers integrin internalization and cell detachment. Therefore, PAI-1 and LRP function as a switch between cell adhesion and integrin disengagement/receptor internalization (Figure 7).
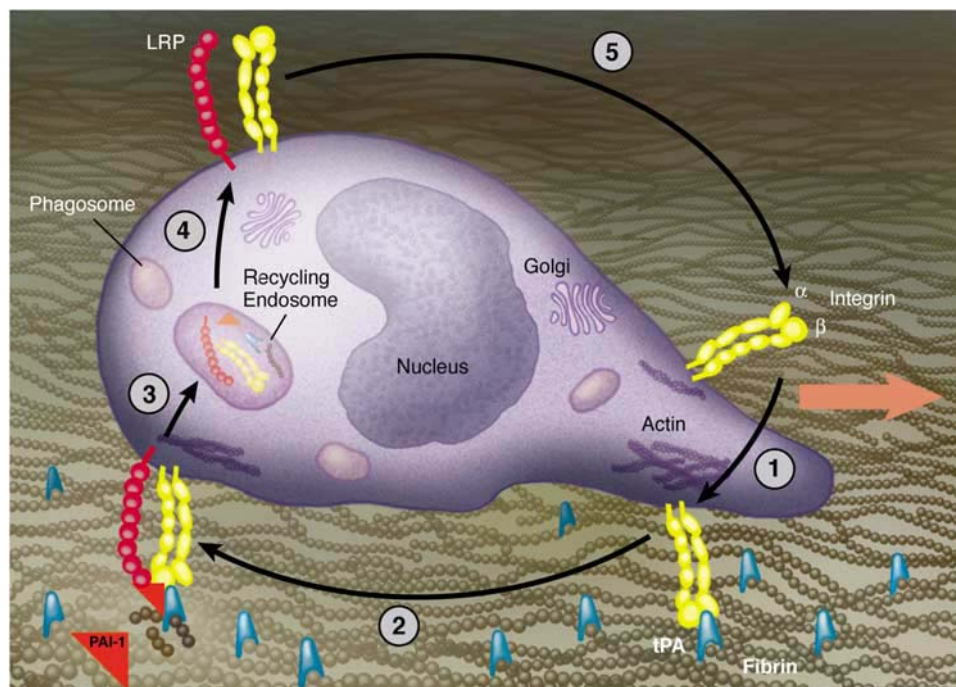

Figure 7 A model for macrophage migration within an inflammatory environment. Stimulated macrophages attach to the extracellular matrix via the tPA/fibrin binary complex (Step 1) and move forward. Fibrin at the trailing edge is partially degraded by tPA-mediated fibrinolysis. PAI-1-neutralized tPA by which it links the adhesion complex to LRP (Step 2). LRP engagement results in a switch from cell adhesion to cell detachment and integrin internalization (Step 3). The internalized Mac-1/LRP complex is returned to the cell surface (Step 4), moves to the cell leading edge (Step 5), and the cycle starts over again. Thus, PAI-1 and LRP, functioning as a master switch, ensure cell attachment, detachment, and integrin recycling to proceed properly in time and in space, leading to efficient cell migration.

In summary, our study emphasizes the critical role of an ordered transition, both in time and in space, between the individual steps of the cell migratory process (adhesion, forward movement, cell detachment, and receptor recycling) in efficient cell migration. Moreover, our results support a model in which efficient migration of inflammatory macrophages within an inflammatory environment depends on the cooperation of at least three physiologically prominent systems (integrin-mediated adhesion, fibrinolysis, and endocytosis). Therefore, the information provided in this study could be useful to our understanding of the molecular mechanism that regulates the transition from innate to adaptive immunity and that prevents the development of various inflammatory diseases.

Materials and methods

Reagents
Mac-1/293 cells, LRP-specific antibodies (R2629 and mAb 5A6), and PAI-1($R^{76}E$) were prepared as described previously (Strickland *et al*, 1990; Zhang and Plow, 1996; Stefansson *et al*, 1998). The DDE complex was provided by Dr Medved (Baltimore, MD). NIF was obtained from Dr Plow (Cleveland, OH). Other reagents, including mAb 11H4 against LRP, were obtained from commercial sources (see Supplementary data for details).

Mice
The WT and deficient mice were all in the C57BL6J background, 8–13 weeks old. The Mac-1$^{-/-}$ mice were kindly provided by Dr Ballantyne, Baylor College of Medicine. Mice deficient in uPA, uPAR, tPA, or PAI-1 were kindly provided by Dr Bugge (Bethesda, MD). The GFP transgenic mice were from the Jackson Laboratory. The LRP-floxed mice were prepared as described (Rohlmann *et al*, 1996). Animals were housed in a pathogen-free facility, and all procedures were performed in accordance with Institutional Animal Care and Use Committee approval.

Macrophage migration in vivo
WT and deficient mice were injected i.p. with 1 ml of 5% sterile TG broth. After 4 days, mature macrophages were stimulated by i.p. injection of 200 μl of 5 μg/ml LPS. After 3 h, the leukocyte numbers in the peritoneal lavage were determined by hemocytometer, and the percentages of macrophages were assessed by FACS analysis with mAb F4/80, and by morphological examination.

Macrophage migration on fibrin(ogen)
Peritoneal macrophages stimulated with 10 ng/ml LPS were allowed to migrate across fibrin(ogen)- (10 μg/ml) or fibronectin-coated inserts (10 μg/ml) (5 μm pore) using a 24-well transwell plate (Costar). After 4 h at 37°C, the number of migrated cells was determined by hemocytometer, and cell differential counts were carried out by Cytospin as described above.

For live cell imaging, migration of freshly isolated macrophages from WT and different deficient mice was performed with the Neue LiveCell System (Camp Hill, PA) at 37°C and 5% CO_2. Time-lapse images were taken in 20-s intervals. Image analysis was performed using Metaview (Universal Imaging). Cell retraction was expressed as the percentages of motile cells that have successfully detached their trailing edges within 20 min, based on analyses of the entire view field taken at ×20 magnification. Migration speeds and the lengths of the trailing tails were measured using MetaMorph (Universal Imaging).

Genetic deletion of LRP in macrophages
Peritoneal macrophages from WT and the LRP-floxed mice were infected with adenoviruses expressing either GFP alone (Ad5CMV-GFP) or GFP and Cre (Ad5 CMV-Cre-GFP). At 20 h after infection, LRP expression was evaluated by two-color FACS analysis, using GFP (marker of infection) and antibody R2629 (LRP) and RPE-anti-rabbit IgG.

Binding assays
ELISA were conducted as described previously (Li and Zhang, 2003). The IP experiments were performed as described (Li and Zhang, 2003) and internalization of surface Mac-1 was determined by FACS analysis. Cell adhesion to the fibrin DDE complex was carried using our published methods (Li and Zhang, 2003). Please see Supplementary data for details.

Confocal laser scanning fluorescence microscopy
LPS-stimulated peritoneal macrophages were stained with R2629 (for LRP) and M1/70 (for Mac-1), and their corresponding secondary antibody conjugated with Alexa 488 or Alexa 568 (Molecular Probes). Nonimmune IgGs were used as controls. The stained macrophages were analyzed using a Bio-Rad Radiance 2000 Confocal Laser Scanning Fluorescence Microscope System. The extent of colocalization between Mac-1 and LRP was analyzed by calculating the volumes of Mac-1 and LRP staining that have the same x–y–z coordinates (overlapping) and then divided by the total volumes of the Mac-1 and LRP staining, as instructed by the Volocity software (Improvision). Three-dimensional reconstruction of the fluorescence confocal images was carried out by deconvolution using Volocity based on the manufacturer's instruction.

Fluorescence lifetime imaging microscopy
Images were acquired using a BioRad Radiance 2000 multiphoton microscope, equipped with a high-speed Hamamatsu MCP detector. Excitation at 800 nm was empirically determined to excite Alexa 488, but not Alexa 568. Donor (Alexa 488) fluorophore lifetimes were fit to two exponential decay curves to calculate the fraction of fluorophores within each pixel that interact with an acceptor. As a negative control, Alexa 488 lifetime was measured in the absence of acceptor (Alexa 568), which showed lifetimes equivalent to FITC–IgG alone or in solution (von Arnim *et al*, 2004).

Statistical analysis
Student's *t*-test was used to analyze significance between two groups. A *P*-value of less than 0.05 was considered significant.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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