Inhibition of calpain stabilises podosomes and impairs dendritic cell motility

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Summary

Podosomes, highly dynamic adhesion structures implicated in cell motility and extracellular matrix degradation, are characteristic of certain cells of the myeloid lineage and a limited range of other cell types. The nature and the mechanisms that regulate their high turnover are unknown at present. The cysteine protease calpain is involved in the regulation of cell migration in part by promoting either formation or disassembly of adhesion sites. Despite the fact that many known substrates of calpain are also structural components of the podosome complex, no studies have yet demonstrated that calpain participates in the regulation of podosome dynamics. In the present work, we show that inhibition of calpain in primary mouse dendritic cells leads to enhanced accumulation of actin filaments, the Wiskott Aldrich Syndrome protein (WASP), β_2 integrins, talin, paxillin and vinculin in podosomes. This accumulation of components is associated with stabilisation of podosome turnover, overall reduction in velocity of cell locomotion and impaired transmigration across an endothelial monolayer. We also demonstrate that calpain cleaves the podosome components talin, Pyk2 and WASP in dendritic cells. In summary, our results provide evidence that calpain regulates podosome composition and turnover and that this process is required for efficient migration of dendritic cells.

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Introduction

Cell locomotion involves a highly regulated cycle of assembly and disassembly of cell adhesions with the surrounding matrix and neighbouring cells (Adams, 2002). In most cell types, focal complexes are assembled at the first contact points between dynamic extensions of the cell membrane (filopodia and lamellipodia) at the leading edge of the cell and the substratum, whereas more stable types of adhesions (focal adhesions and their variants), linked to the actomyosin-based contractility machinery, allow cell traction, contraction and the release of the rear of the advancing cell body (Ridley et al., 2003).

In addition to these classical adhesion structures, certain cells of the myeloid lineage; macrophages (Linder et al., 1999), dendritic cells (DCs) (Binks et al., 1998) and osteoclasts (Calle et al., 2004b; Zambonin-Zallone et al., 1988) assemble highly dynamic conical structures named podosomes. These structures resemble adhesion sites elaborated by Src-transformed fibroblasts (Marchisio et al., 1987; Abram et al., 2003), as well as agonist-activated endothelia (Moreau et al., 2003; Osiak et al., 2005) epithelia (Spinardi et al., 2004) and smooth muscle cells (Kaverina et al., 2003). Although podosomes share many molecular components in common with focal complexes and focal adhesions, they have specific elements and a distinctive organisation (Buccione et al., 2004; Linder and Aepfelbacher, 2003). They consist of a core of actin filaments surrounded by a ring made up of vinculin, paxillin, talin, fimbrin, gelsolin, vimentin (Marchisio et al., 1984; Marchisio et al., 1987) and other adaptor molecules (Abram et al., 2003; Oda et al., 2001), that are linked to adhesion molecules of the integrin family (Gaidano et al., 1990; Linder and Aepfelbacher, 2003). In addition, unlike focal complexes, podosomes contain members of the WASP family (Burns et al., 2001; Spinardi et al., 2004).

In myeloid cells, podosomes localise behind the leading edge of migrating macrophages, DCs and osteoclasts (Jones et al., 2002; West et al., 2004; Lakkakorpi and Vaananen, 1991) and are thought to play an important role in locomotion and extracellular matrix degradation (Linder and Aepfelbacher, 2003; Brunton et al., 2004). In osteoclasts they also form concentric rings that define the sealing zone (Destaing et al., 2003). They are very dynamic; changing in size and position with a minimum half-life of 2 minutes in osteoclasts (Kanehisa et al., 1990) and 1 minute in macrophages (Evans et al., 2003) and between 30 seconds and 10 minutes in DCs (Calle et al., 2004a) (our unpublished data) depending upon migratory status. Since new podosomes are constantly generated in moving cells, regulation of these adhesions implies not only a rapid assembly of their components but also fast disassembly, possibly to allow effective progression of the leading edge during cell migration. The nature and the mechanisms of regulation of this cyclical turnover are unknown at present.

Calpains are a family of intracellular calcium-dependent

cysteine proteases involved in the regulation of signalling pathways by degrading and/or generating biologically active fragments of specific target proteins (Carragher and Frame, 2002; Franco and Huttenlocher, 2005). During cell locomotion, calpains have been shown to promote the disassembly of focal complexes (Bhatt et al., 2002) and focal adhesions (Carragher et al., 2001) by cleaving certain components of these adhesion structures. Other studies have found that calpain participates in the formation of integrin-mediated adhesion sites (Rock et al., 2000; Yan et al., 2001). Although many components of cell adhesions are substrates of calpain in vitro, it remains unclear whether these proteins are cleaved in a cellular context and what the biological significance of this cleavage may be. Many known substrates of calpain are also structural components of the podosome complex, which makes this class of protease a strong candidate as a regulator of their formation and/or disassembly.

In the present work, we have investigated whether calpain plays a role in podosome dynamics and the possible consequences for DC motility. Our results provide evidence that in DCs, calpain regulates podosome turnover and composition and this process is required for efficient cell adhesion and migration.

Results

Calpain activity is required for disassembly of preformed podosomes in migrating DCs

Using immunofluorescence and western blotting we found that DCs expressed both calpain 1 and calpain 2 and that they can be found in a zone surrounding the actin core (Fig. 1). In the case of calpain 1, we also detected colocalisation with actin filaments at the edges of the actin core, mainly in smaller podosomes located towards the rear of the cluster (Fig. 1A, arrowheads). To test whether calpains play a role in the formation and/or organisation of podosomes in migrating DCs we incubated DCs with the peptide inhibitors ALLM, ALLN or with a 27-amino-acid peptide containing a sequence encoded by exon 1B of the highly specific endogenous calpain inhibitor calpastatin, using as a negative control a scrambled peptide of the calpastatin sequence. The calpain inhibitors, but not inhibitors of cathepsins or the proteasome, induced a significant drop in calpain activity in DCs (Fig. 2A) in a dose-dependent manner (data not shown). The percentage of plated cells with podosomes was not significantly altered upon the inhibition of calpain activity with any of the calpain inhibitors used (Fig. 2B), suggesting that calpain activity is not required for podosome assembly. On the contrary, inhibition of calpain in DCs with preformed podosomes resulted in the formation of more robust podosomal structures. Untreated mouse DCs assembled rings of β_2 integrin (Fig. 3A), vinculin (Fig. 3A), talin (Fig. 3J) and paxillin (supplementary material, Fig. S1) around the actin core as shown for human DCs (Burns et al., 2001). Inhibition of calpain induced an exaggerated accumulation of β_2 integrin and vinculin in the podosome complex (Fig. 3B-E) resulting in a 40.0%, 43.2% and 48.1% increase in the fluorescent intensity of β_2 staining (Fig. 3F) and a 68.5%, 74.6% and 90.2% increase in the fluorescent intensity of vinculin staining (Fig. 3G) in DCs treated with ALLM, ALLN and calpastatin peptide, respectively. The podosome core also increased in F-actin content and gross dimension as detected by immunofluorescence (Fig. 3A-E) resulting in an increase of 52.11%, 56.3% and 49.0% in the fluorescent intensity of F-actin (Fig. 3H) and a 91.1%, 107% and 105.5% increase in diameter size (Fig. 3I) in DCs treated with ALLM, ALLN and calpastatin peptide, respectively. The integrin-associated proteins talin (Fig. 3J-N) and paxillin (supplementary material, Fig. S1) also accumulated around the actin core of podosomes as a result of calpain inhibition. Treatment of DCs with ALLM, ALLN and calpastatin peptide induced a 79.9%, 81.3% and 110.9% increase in talin fluorescent intensity, respectively (Fig. 3O). Removal of the calpain inhibitors restored DC podosomal organisation to normal after overnight culture in untreated culture medium (data not shown). Inhibition of other cysteine proteases such as cathepsins or inhibition of the proteasome resulted in the disassembly of podosomes in DCs (Fig. 2B and Fig. S2 in supplementary material), confirming the specificity of the findings to calpain inhibition with ALLM and ALLN, which may also inhibit cathepsins at high concentrations. Taken together with the data obtained using the highly specific calpastatin peptide, our results show that calpain activity is required for the breakdown of DC podosomes because inhibition of this protease resulted in an increase in the total

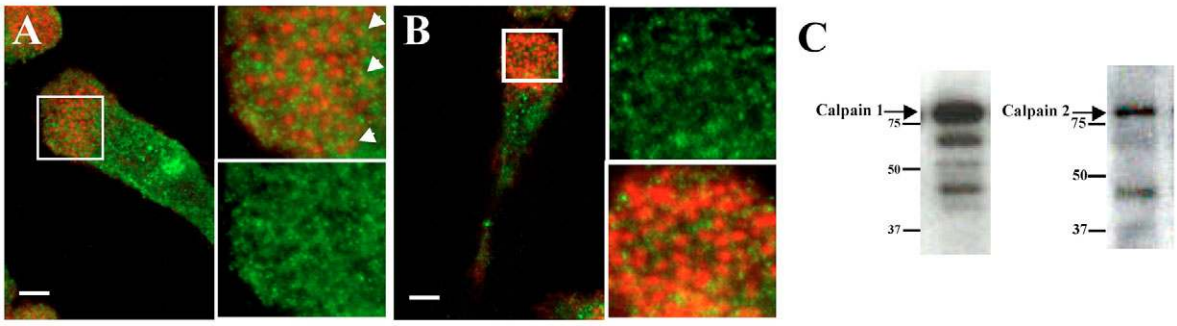

Fig. 1. Calpain 1 and 2 are expressed in DCs. DCs plated overnight on poly-L-lysine-coated glass coverslips were fixed with 3% paraformaldehyde, permeabilised with 0.05% Triton X-100 and double stained to detect calpain 1 (green) and actin (red) (A) or calpain 2 (green) and actin (red) (B). Arrowheads in A indicate the colocalisation of calpain 1 and actin at the edges of the podosome core at the rear of the podosome cluster. Magnifications of the boxed areas in A and B with calpain staining and double staining are shown on the right. Bars, 10 μm. (C) Western blots of calpain 1 and calpain 2 in total lysates of DCs. The positions of molecular size markers (in kDa) are indicated.

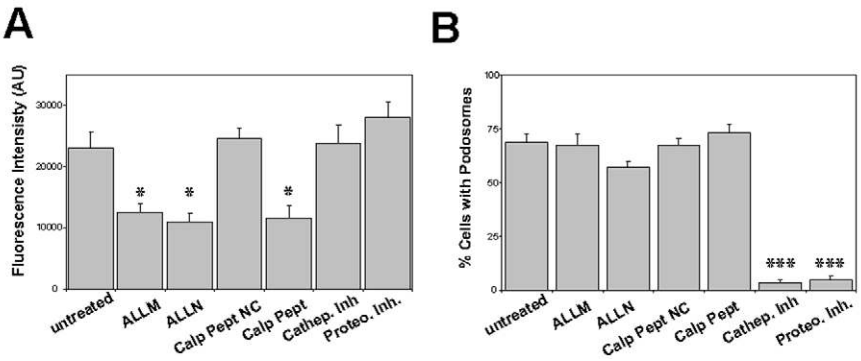

Fig. 2. Calpain regulates podosome disassembly in DCs. (A) Calpain activity in the presence of inhibitors was detected. As expected, inhibition of calpain activity was found using the calpain inhibitors ALLM (50 μM), ALLN (50 μM) or the calpastatin peptide (Calp Pept, 50 μM) but not using cathepsin inhibitor 1 (Cathep. Inh,10 μM), proteosome inhibitor 1 (Proteo. Inh, 10 μM) or the scrambled version of the calpastatin peptide (Calp Pept NC, 50 μM). (B) The percentage of DCs with podosomes was not affected by treatment with calpain inhibitors. Inhibition of cathepsin B and proteosome resulted in DC podosome loss. Results are mean values ± s.e.m. from 50 cells with three coverslips examined per experiment. **P*<0.05 and ****P*<0.005 compared with values in untreated cells (Student's *t*-test).

F-actin within the actin core and the accumulation of associated podosomal proteins.

Calpain regulates adhesion turnover and motility of DCs

Podosomes occupy the most prominent area of contact of DCs to the substratum as shown by interference reflection microscopy (IRM) and are clearly distinct from focal complexes and focal adhesions (Fig. S3 in supplementary material). Using time-lapse IRM, we tested whether the formation of more robust podosomes by the inhibition of calpain resulted in an increase in the stability of these adhesions. Treatment of DCs with the calpain inhibitors ALLM, ALLN or the calpastatin peptide reduced the turnover index of podosomes by 84.9%, 66.5% and 76.6%, respectively (Fig. 4A). This increased stability of DC adhesions correlated with a decrease in cell speed by 66.6%, 73.3% and 66.6% after treatment with ALLM, ALLN and the calpastatin peptide, respectively (Fig. 4B and Movies in supplementary material). These results show that calpain activity is essential for the disassembly and turnover of podosomes and this process is essential for translocation of migrating DCs.

Calpain activity is essential for efficient adhesion in spreading DCs

To study the possible requirement of calpain activity for the assembly of new adhesions, especially podosomes, we analysed the effect of calpain inhibition on freshly seeded DCs. As a result of pre-treatment of DCs with ALLM and ALLN, freshly seeded cells failed to spread to the same extent as untreated cells (Fig. 5A) and the adhesion efficiency was decreased by 42.3% and 34.6%, respectively (Fig. 5B).

Using immunocytochemistry, we found that in the initial 10 minutes of spreading, cells treated with ALLM and ALLN were able to assemble centrally located podosomes and focal complexes similarly to untreated cells (Fig. 6A-C). However, with increasing time, podosomes grew in size and failed to reorganise in the cell body compared with untreated cells. Thus at 15 minutes, in untreated cells podosomes remained clustered and displayed some asymmetry of distribution towards the extending lamellae (Fig. 6D, arrowhead). ALLM- and ALLN-treated cells failed to generate lamellar extensions but instead displayed spikes around the whole periphery whereas podosomes appeared centrally located and richer in actin filaments (Fig. 6E,F). By 2 hours, the majority of untreated DCs became elongated and polarised with a cluster of podosomes behind the leading edge (Fig. 6G). In ALLM-treated cells, podosomes were larger with more robust actin cores and more defined β_2 integrin and vinculin rings compared to untreated cells (Fig. 6H). Similarly, ALLN treated cells remained round with a central cluster of robust podosomes rich in actin, β_2 integrin and vinculin (Fig. 6I).

The accumulation of actin, β_2 integrin subunits and vinculin in podosomes over time following inhibition of calpain suggests that podosomal components are sequestered and not released to contribute to the formation of new adhesions that would support lamellar extensions.

Calpain activity is required for endothelial transmigration of DCs

Migration across the endothelium is a requirement for DCs to settle in peripheral tissues and, when pathogens are encountered, to leave the resident tissue and reach the lymph nodes for antigen presentation (Imhof and Aurrand-Lions, 2004). Hence, we decided to test whether the regulation of DC adhesion turnover by calpain had any effect on the motility of DCs plated on the more relevant substratum provided by a monolayer of endothelial cells. Treatment of DCs with calpain inhibitors resulted in impaired transmigration of DCs across a monolayer of SVEC 4-10 endothelial cells. Calpain inhibition with both ALLM and ALLN decreased the percentage of transmigrated DCs across the monolayer by 68.0% and 90.2%, respectively (Fig. 7A). Consequently, there was a 4.6-fold increase in the percentage of cells left on top of the monolayer with both treatments. In addition, the area of untreated DCs that had spread under the endothelial monolayer following transmigration was significantly higher than that of the few ALLM (1.5-fold) and ALLN (1.9-fold) treated DCs (Fig. 7B,C).

The Wiskott Aldrich Syndrome protein (WASP), Pyk2 and Talin are cleaved by calpain in migrating DCs

In order to study which podosomal proteins might be cleaved by calpain in migrating DCs we analysed defined podosome

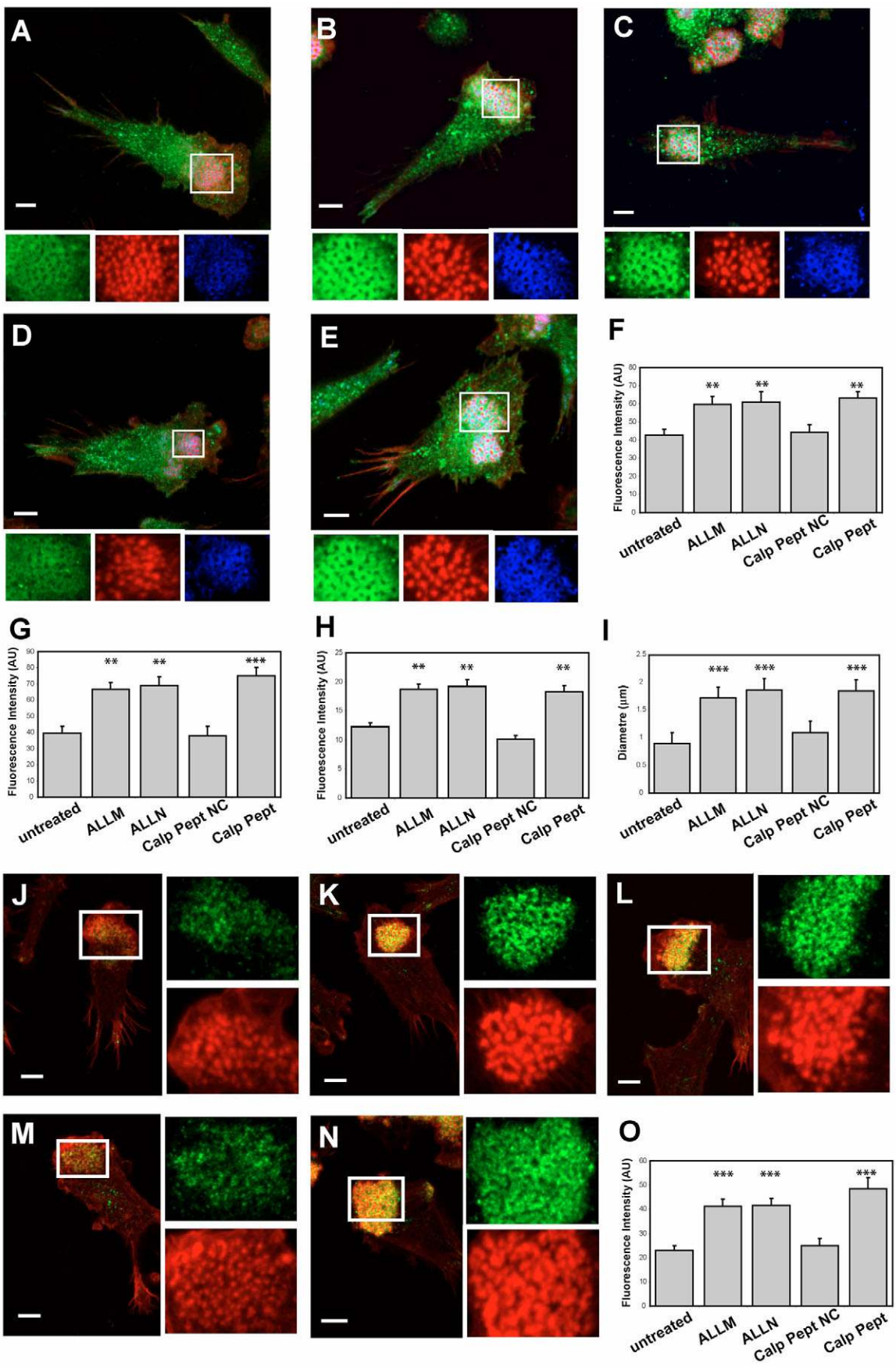

Fig. 3. See next page for legend.

Fig. 3. Treatment of DCs with calpain inhibitors results in accumulation of β_2 integrin subunits, actin, vinculin and talin in podosomes. DCs plated overnight on poly-L-lysine-coated glass coverslips were left untreated or were treated with ALLM, ALLN, calpastatin peptide or the scrambled version of the calpastatin peptide (Calp Pept NC) and then fixed with 3% paraformaldehyde, permeabilised with 0.05% Triton X-100 and triple stained to detect β_2 integrin subunit (green), actin (red) and vinculin (blue) (A-E). Inhibition of calpain with ALLM (B), ALLN (C) or the calpastatin peptide (E) resulted in an exaggerated accumulation of β_2 integrin subunits, vinculin and actin in DC podosomes, compared with untreated cells (A) or cells treated with the scrambled version of the calpastatin peptide (D). β_2 integrin subunit, actin and vinculin single staining from the boxed areas in main panels is shown magnified below. Quantification of the fluorescent intensity of the staining of β_2 integrin (F), vinculin (G) and actin (H). Sizes of the actin core of podosomes of untreated and treated DC (I). Alternatively, DCs were stained to detect talin (green) and actin (red) (J-N). Inhibition of calpain with ALLM (K), ALLN (L) or the calpastatin peptide (N) induced an exaggerated accumulation of talin in DC podosomes, compared with untreated cells (J) or cells treated with the scrambled version of the calpastatin peptide (M). Single staining is shown to the right of panels J-N. (O) Quantification of the fluorescent intensity of the staining of talin. The micrographs are representative of the cytoskeletal organisation of DCs detected in three independent experiments. Bars, 10 μm. The graphs illustrate the mean percentage of cells ± s.e.m. from three experiments with 20 cells per treatment, per experiment. $^{**}P<0.01$, $^{***}P<0.005$ compared with values in the untreated cells (Student's *t*-test).

components that are known to be calpain substrates. Using western blotting, we probed the possible presence of cleavage fragments in suspended cells (non migrating and podosomes absent), migrating cells which had been plated overnight on coverslips and migrating cells plated under the same conditions but treated with the calpain inhibitors ALLM or ALLN. In total cell lysates of migrating cells we detected the cleavage of WASP, talin, Pyk 2 and vinculin (Fig. 8). Other proteins, such as Src did not show any cleavage fragments (Fig. 8). The cleavage products of WASP of approximately 40 kDa and 45 kDa, the 190 kDa, 90 kDa and 70 kDa cleavage products of talin and a 50 kDa band of Pyk 2 were significantly reduced in the presence of ALLM and ALLN, indicating that of those studied, only these three proteins were substrates for calpain in migrating DCs. The fact that treatment of DCs with calpain inhibitors prevents the formation of the cleavage products of talin, Pyk2 and WASP but the amount of intact protein remain largely unchanged indicates that calpain mediates the limited proteolysis of these proteins rather than extensive degradation. Incubation of FLAG-WASP generated using Promega TNT® Quick coupled in vitro translation system, with pure calpain resulted in the generation of WASP fragments with a similar size to the ones detected in lysates of migrating DC (Fig. S4 in supplementary material). Taken together, these data provide a strong link between reduced podosome turnover (Fig. 4A) and reduced cleavage of Pyk 2, WASP and talin caused by the inhibition of calpain activity.

WASP is required for formation of dynamic DC podosomes and location of WASP in DC is regulated by calpain activity

We have previously shown that in the absence of WASP, murine DCs fail to form podosomes and focal contacts are assembled instead (Calle et al., 2006). We have also shown that expression of eGFP-WASP in WASP-null DCs results in reconstitution of podosomes and that eGFP-WASP localises to the core of these adhesive structures. Adhesion turnover of WASP null DCs was 54.6% lower compared with SV129 (wild-type) DCs (Fig. 9). This result was expected because focal-adhesion-like structures in other cell types are less dynamic than podosomes. Expression in WASP-null DCs of eGFP-WASP but not of eGFP alone induced a 82.28% increase in adhesion turnover so that it reached levels equivalent to wild-type DCs. In addition, treatment of eGFP-WASP-expressing WASP-null DCs with the calpain inhibitors ALLM or ALLN resulted in a 64.2% and 72.0% reduction in the adhesion turnover. Time-lapse live-cell imaging using confocal microscopy showed that WASP-null cells expressing eGFP-WASP and treated with calpain inhibitors accumulated substantial eGFP-WASP in podosomes correlating with the stability of these adhesions (Fig. 9 and Movies in supplementary material).

Discussion

The initial response of a cell to a migration stimulus is to polarise and extend protrusions that then define the direction of locomotion. These protrusions take the form of broad lamellipodia, and more often than not are seen in association

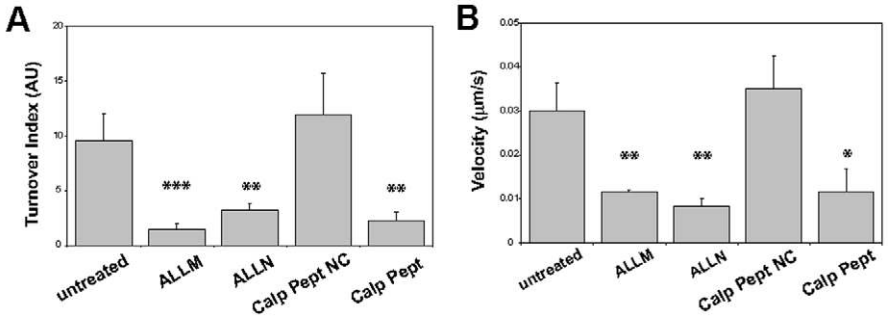

Fig. 4. Adhesion turnover and motility of DCs are regulated by calpain. (A) DCs were plated on poly-L-lysine-coated coverslips overnight and then mounted in viewing chambers to measure the adhesion turnover index using IRM time-lapse video. Values are mean ± s.e.m. of ten cells per treatment group from three experiments. (B) Phase-contrast time-lapse videos were recorded to measure the velocity of DCs in the viewing chambers. Values are mean ± s.e.m. percentage of 20 cells per treatment group from three experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ compared with values in untreated cells (Student's *t*-test).

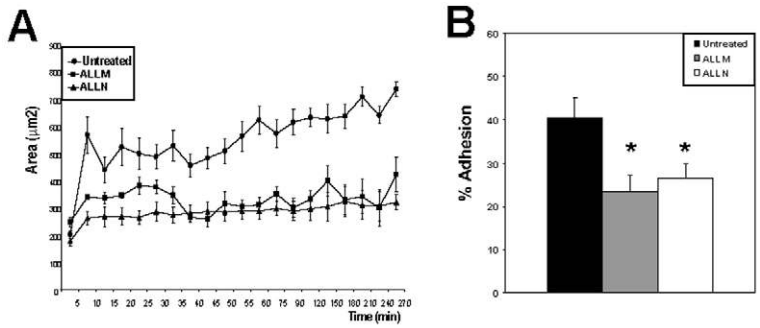

Fig. 5. Calpain regulates spreading and adhesion of DCs. Using phase-contrast video time-lapse microscopy, untreated and ALLM or ALLN treated cells were filmed for 240 minutes while spreading on poly-L-lysine coated dishes. (A) Area of cell spread on the substratum measured using motion analysis software. The graphs illustrate the mean percentage of cells ± s.e.m. from three experiments. The area of treated cells was significantly different to that in untreated cells ($P<0.005$) by ANOVA analysis. (B) Percentage of adhesion of DCs on poly-L-lysine-coated wells 30 minutes after plating. The graphs illustrate the mean percentage of cells ± s.e.m. from three experiments *$P<0.05$ compared with untreated cells (Student's *t*-test). ALLM and ALLN treatments resulted in decreased area of spread and adhesion efficiency.

with spike-like protrusions called filopodia beyond the leading edge of the cell. Both structures are formed as a result of actin polymerisation and are stabilised by adhesion to either the extracellular matrix or to neighbouring cells by transmembrane receptors that are also linked to the actin cytoskeleton (Ridley et al., 2003). Microtubules serve to stabilise this initial response (Small et al., 2002) and cooperate with the actin cytoskeleton in regulating adhesion integrity (Krylyshkina et al., 2003). For cell translocation over a substratum, a mechanism to regulate disassembly of adhesions must also be in place to allow forward progression of the cell.

Calpain is thought to play an important role as a regulator of immunity by participating in the control of adhesion of immune cells such as T cells (Glading et al., 2002; Stewart et al., 1998). Certain haematopoietic cells, including macrophages, DCs and osteoclasts form specialised actin cytoskeletal structures known as podosomes, that are important for adhesion to underlying substrata, and probably for efficient cell migration. The mechanisms regulating the high turnover of podosomes are largely unknown. The presence of substrates of calpain in the podosome complex suggested this cysteine protease might play a role in the formation and/or disassembly

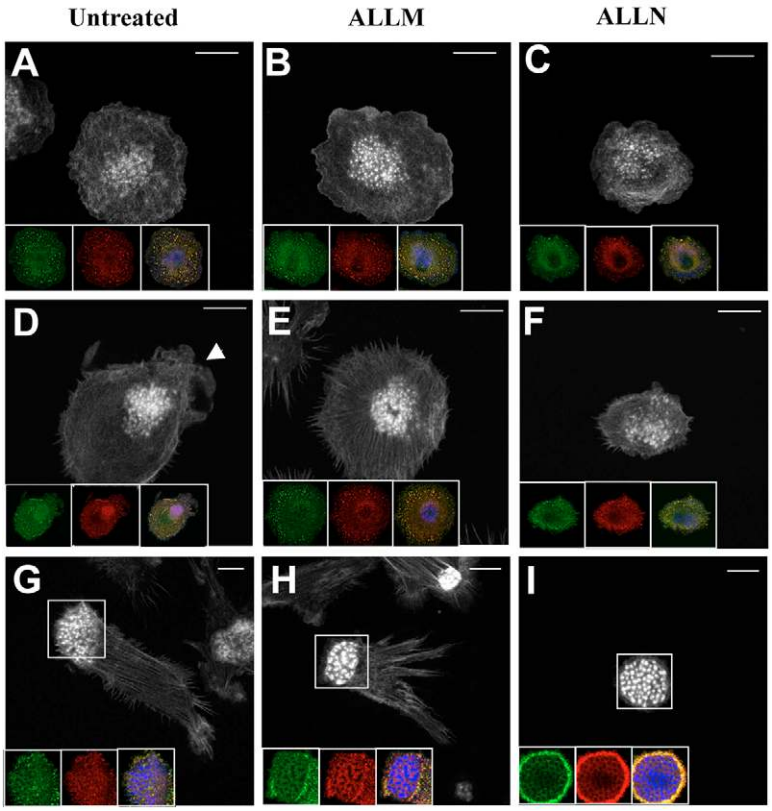

Fig. 6. Inhibition of calpain results in accumulation of podosome components in spreading DCs. Untreated and ALLM- or ALLN-treated DCs were allowed to spread on poly-L-lysine-coated coverslips for 10 minutes (A,B,C), 15 minutes (D,E,F) or 2 hours (panels G,H,I). At these time points cells were fixed with 3% paraformaldehyde, permeabilised with 0.05% Triton X-100 and stained to detect β_2 integrin subunits, vinculin and actin. The main images show the distribution of actin and inserts the localisation of β_2 integrin subunits (green), vinculin (red) and the colocalisation with actin (blue). Five minutes after plating, untreated cells developed peripheral lamellae and a central cluster of podosomes (A). At 15 minutes, the central cluster of podosomes displayed some asymmetry of distribution towards the extending region of lamellae (D, arrowhead). By 2 hours, the majority of untreated DCs were polarised with a cluster of podosomes behind the leading edge (G). Inhibition of calpain resulted in increased accumulation of β_2 integrin subunits, vinculin and actin in podosomes. At 15 minutes, cells failed to develop major lamellar extensions and only peripheral spikes were seen (E,F). By 2 hours, ALLM- and ALLN-treated cells displayed podosomes richer in β_2 integrin subunits, vinculin and actin (panels H,I). The micrographs are representative of the cytoskeletal organisation of DC detected in three independent experiments. Bars, 10 μm.

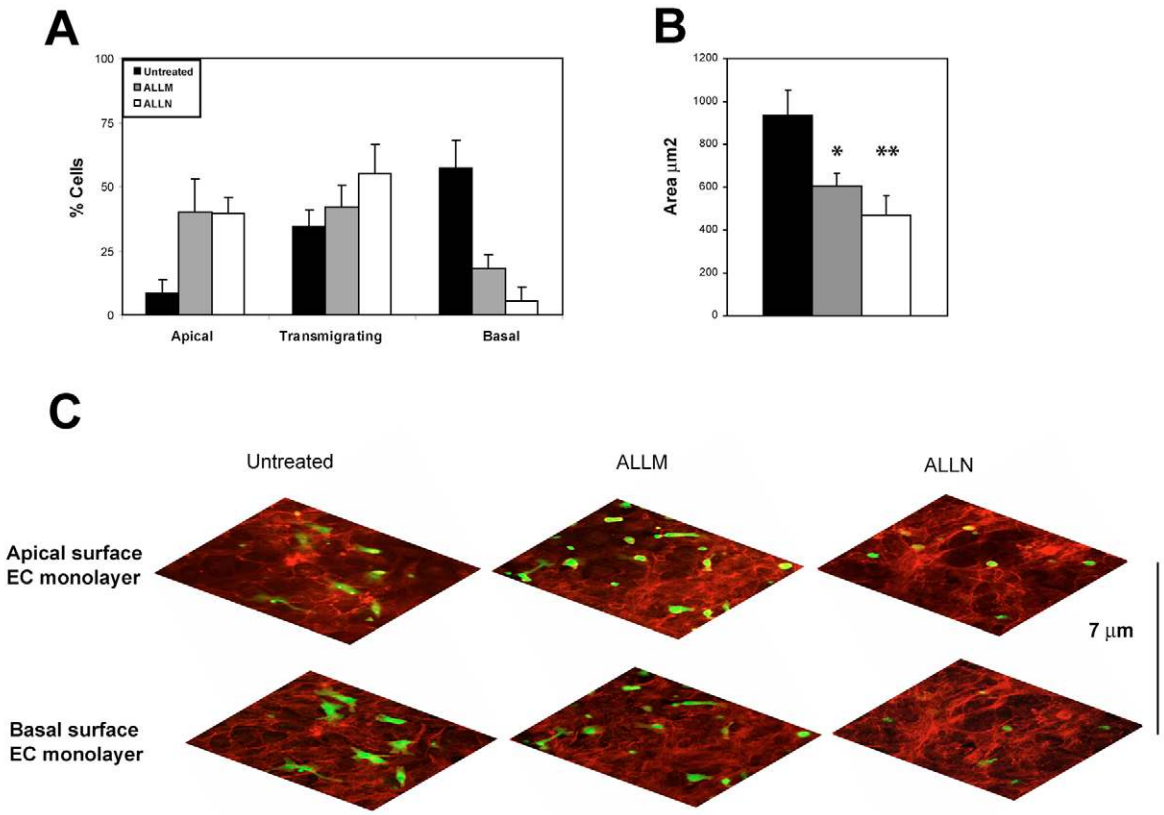

Fig. 7. Calpain activity is required for transendothelial migration of DC. After incubation of CFSE-labelled DCs on a monolayer of SVEC 4-10 endothelial cells for 1 hour, co-cultures were washed once with PBS and fixed with 3% paraformaldehyde, permeabilised with 0.05% Triton X-100 and stained to detect actin filaments. (A) Percentage of DC transmigrated, transmigrating or retained on the apical surface of the monolayer. (B) Spread area of DCs under the monolayer in untreated (black), ALLM-treated (grey) and ALLN-treated (white) cells. Values are mean ± s.e.m. of 50 measurements from three coverslips. (C) Skewed confocal micrographs of optical sections at the apical and basal poles of endothelial cell monolayers. CFSE-labelled DCs are green and the distribution of actin filaments is shown in red. Treatment of DCs with calpain inhibitors reduced the transmigration of DCs across the endothelial cells and reduced the spread area under the endothelial cell monolayer. The graphs illustrate the mean percentage of cells ± s.e.m. from three experiments. $*P<0.05$, $**P<0.01$ compared with levels in untreated cells (Student's *t*-test). The micrographs are representative of the cytoskeletal organisation of DCs detected in three independent experiments.

of podosomes as it does in focal complexes (Bhatt et al., 2002; Bialkowska et al., 2000). As shown in this study, inhibition of calpain in migrating DCs led to an exaggerated accumulation of actin filaments, β_2 integrins, the integrin associated proteins talin, paxillin and vinculin, and the adaptor protein WASP in podosomes indicating a role for calpain in their disassembly, and promoting turnover of their integral components. This was confirmed by interference reflection microscopy, which showed that the rate of DC podosome turnover was dependent on calpain activity. In addition, delayed podosome turnover in DCs resulted in reduced cell locomotion suggesting that the turnover of podosomes is a requirement for DC motility as has been shown for osteoclasts (Goto et al., 2002)

Recently, it has been shown that cleavage of talin by calpain is a limiting factor during adhesion turnover and that disassembly of paxillin and vinculin from adhesion complexes is dependent on this process (Franco et al., 2004). Similarly, in this study we found that inhibition of calpain-mediated talin cleavage was associated with the accumulation of paxillin and vinculin in podosomes. The increased levels of these two components may provide a firmer anchorage for actin filaments in the core to the integrin-associated complex in the podosome periphery, leading to enhanced structural stability. In addition, vinculin has been proposed to directly couple cell adhesion and actin polymerisation by binding to the actin polymerisation nucleating factor, Arp2/3 complex (DeMali et al., 2002). Accumulation of vinculin could therefore directly promote local Arp2/3 complex-mediated actin polymerisation, leading to an increased accumulation of actin filaments in the core. In our study in addition to detecting a fragment corresponding to the talin rod described in previous studies in cells or in vitro, we detect two other fragments whose size corresponds to two described fragments resulting from further cleavage of talin in protease-sensitive regions (Critchley, 2004). The fact that calpain inhibitors block the appearance of these fragments indicates that the initial talin rod fragment may make this fragment sensitive for further proteolysis either by calpain or other proteases. It is possible that in DCs the pattern of talin cleavage by calpain is distinct from others. This variation is not exclusive to talin because, for example, α-actinin has been shown to be cleaved by calpain in some cell types but not in vitro (Goll et al., 1991; Selliah et al., 1996).

We and others have shown that the intracellular adaptor protein WASP plays an essential role in podosome assembly in macrophages (Linder et al., 1999), osteoclasts (Calle et al., 2004b) and DCs (Burns et al., 2001). WASP is specifically expressed in haematopoietic cells and belongs to a larger family of more widely expressed proteins that mediate de novo actin polymerisation (Thrasher, 2002) by interacting with signalling molecules, actin and the Arp2/3 complex (Higgs and Pollard, 2001). We have found that the rapid turnover of calpain-sensitive podosome adhesions in DC is WASP-dependent because the absence of WASP leads to loss of podosomes and formation of more stable focal adhesions that lack WASP. In addition, migrating DCs generate WASP fragments that require calpain activity for their formation. The direct cleavage of WASP by calpain in vitro generates equivalent fragments as the ones detected in migrating DCs. Taken together, these findings suggest that regulation of WASP cleavage by calpain is an important step in podosome disassembly; calpain activity does not seem to be required for the recruitment of WASP to podosomes but rather permits the dissolution of these structures by mediating WASP cleavage. Hence, WASP may play a role not only in the formation but also in the disassembly of podosomes, providing a mechanism that regulates the dynamic nature of podosomes. The nature and biological functions of the calpain-generated WASP fragments previously found in platelets (Mukhopadhyay et al., 2001; Oda et al., 1998) and in DCs as reported here remain unknown. We speculate that certain WASP cleavage products are likely to have a direct impact on the formation and/or organisation of actin filaments, perhaps through disturbed localisation of the Arp2/3 complex (Machesky and Insall, 1998).

Calpain has been shown to regulate integrin clustering in focal contacts in spreading cells (Bialkowska et al., 2000; Rock et al., 2000). In our study, pre-treatment of DCs with calpain inhibitors resulted in a failure to increase the area of adhesive contact with the substratum leading to low adhesion efficiency. However, this was not due to failure of podosome formation. Podosomes could still assemble in the presence of calpain inhibitors and over time during spreading, these adhesion structures were significantly more robust than in untreated cells, containing more actin filaments, β_2 integrins and vinculin. These results suggest that calpain activity in spreading cells maybe be required to release components from the initial podosome attachments and allow generation of new adhesive structures that would sustain the extension of the cell body.

In a more physiological system, we tested whether this would translate into deficiencies in migration of DC through endothelial monolayers, which is an essential event during the life of DCs. Once again, we showed that inhibition of cytoskeletal remodelling by calpain resulted in inefficient migration and spreading of DCs.

In summary, we have shown that calpain regulates the protein composition and structure of podosomes, and is crucial for normal podosome turnover in DCs. It is therefore essential

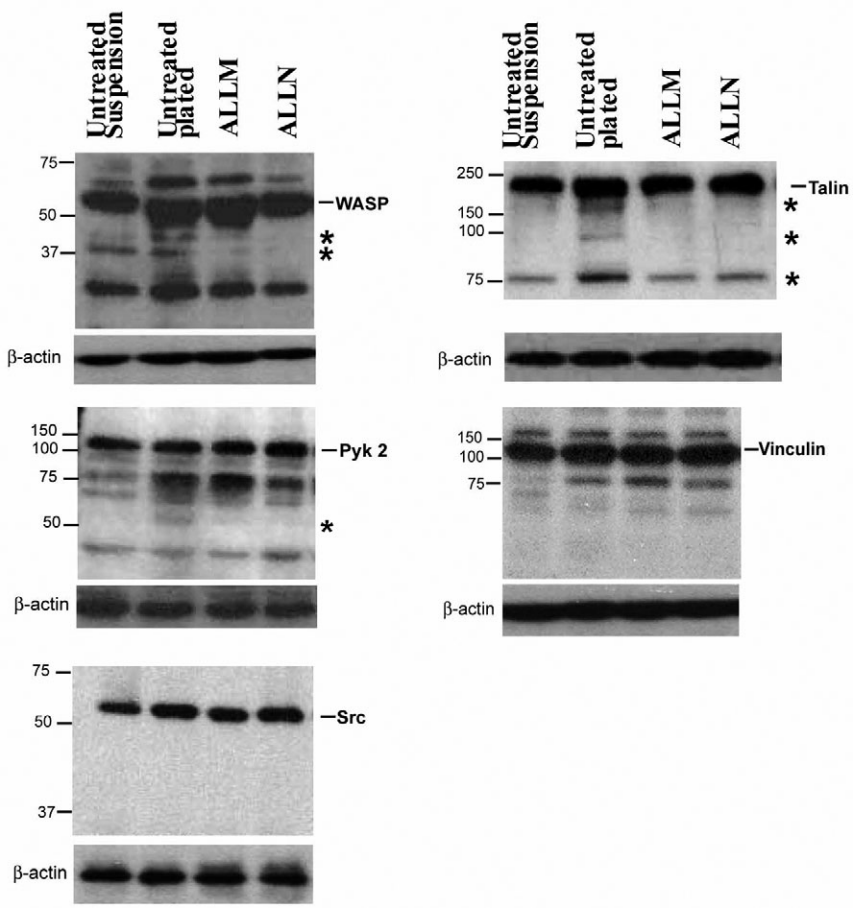

Fig. 8. Calpain cleaves the podosome proteins WASP, Pyk2 and talin. Total lysates of untreated and ALLM- or ALLN-treated plated DCs were separated by SDS-PAGE and western blotted. Immunoblotting with the corresponding antibodies showed that WASP, Pyk2 and talin but not vinculin or Src were cleaved by calpain. Two calpain cleavage products of WASP of ~40 kDa and a 45 kDa (asterisks) were detected. One cleavage product of Pyk2 (50 kDa, asterisk) and three of talin (70 kDa, 90 kDa and 190 kDa, asterisks) were detected.

for effective motility of these cells, such as during migration across endothelial surfaces. In addition, we provide evidence showing that the regulation of DC motility by calpain involves the generation of cleavage fragments of the podosomal components talin and WASP.

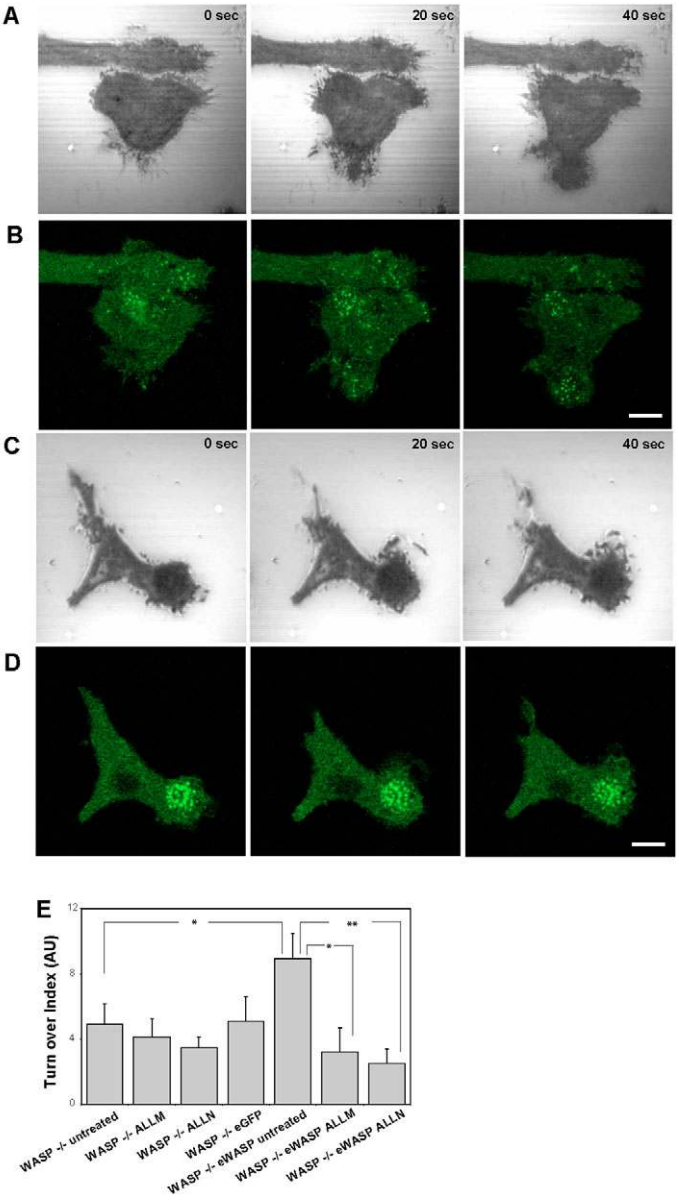

Fig. 9. WASP is required for formation of dynamic DC podosomes and location of WASP in DC is regulated by calpain activity. WASP-null DCs expressing eGFP-WASP were plated on poly-L-lysine-coated coverslips overnight and then left untreated (A,B) or treated with the calpain inhibitor ALLM (C,D) and mounted in viewing chambers to measure adhesion turnover index using confocal microscopy. Micrographs of the interference reflection signal (A,C) and eGFP-WASP location (B,D) were taken 20 seconds apart. Bars, 10 μm. (E) Quantification of adhesion turnover of WASP-null DCs expressing eGFP constructs in the presence or absence of calpain inhibitors. The graphs illustrate the mean percentage of cells ± s.e.m. of 20 cells per treatment group from three experiments. Significant differences were observed at $*P<0.05$ and $**P<0.01$ as indicated.

Materials and Methods

Animals

Pathogen-free SV129 mice purchased from Harlam and WASp-null mice on a SV129 background (Snapper et al., 1998) originally obtained from Dr Fred Alt (Harvard Medical School, Boston, MA) were bred in our own colony in pathogen free conditions.

Cell culture

DCs were generated from mouse spleens as previously described (West et al., 1999). Briefly, spleens from 6- to 8-week-old SV129 mice were homogenised through a cell strainer to obtain a cell suspension. Cells were washed twice with RPMI (Sigma, UK) containing 1% heat-inactivated foetal bovine serum (FBS) and then resuspended in RPMI supplemented with 10% FBS, 1 mM pyruvate (Sigma, UK), 1× non-essential amino acids (Sigma, UK), 2 mM glutamine (Sigma, UK), 50 βM 2-ME (Gibco BRL), 20 ng/ml recombinant mouse GM-CSF (R&D Systems) and 1 ng/ml recombinant human TGF-β (R&D Systems) and plated at a density of 2×10^6 cells/ml in 75 cm^2 culture flasks at 37°C in a 5% CO_2 atmosphere. After 5 days of culture, 5 ml fresh medium were added per flask and at day 8, the cells in suspension were collected, replated and kept in suspension in fresh medium. After a total of 17-18 days ex vivo, 80-90% of the cells in culture were DCs as determined by the expression of CD11c and DEC205 by FACS analysis (data not shown). Cell viability before experimental assays was tested by Trypan Blue exclusion. In addition, we validated the lack of toxicity of inhibitors used in our study by FACS using Propidium Iodide exclusion to test necrosis and cell cycle analysis to test apoptosis. In all cases, cell viability was between 85% and 95%.

The mouse lymphoid microvascular endothelial immortalised cell line, SVEC 4-10 (O'Connell and Edidin, 1990) was obtained from the American Type Culture Collection and cells were cultured using DMEM (Sigma, UK) supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere.

Reagents and antibodies

Antisera to vinculin, paxillin and talin antibodies were purchased from Sigma, UK. Anti-β_2 integrin subunit antibody was purchased from Pharmingen. Antibodies to calpain 1 and calpain 2 were purchased from Triple Point Biologics, USA. Alexa Fluor 488- or Cy5-conjugated anti-mouse IgG antibody and Alexa Fluor 568 phalloidin were obtained from Molecular Probes. FITC-conjugated anti-rat antibody was purchased from Sigma, UK. Anti-WASP antibody was purchased from Upstate Biotechnology. Horseradish peroxidase (HRP)-labelled anti-mouse and anti-rat antibodies were purchased from Dako. Calpain inhibition studies were performed using calpain inhibitor I (ALLN), calpain inhibitor II (ALLM) and the cell-permeable calpastatin peptide, all purchased from Calbiochem, UK. As a negative control for the effect of the calpastatin peptide we used the scrambled version of the peptide from Calbiochem, UK. Inhibition of cathepsins and the proteasome was achieved by treatment of DC with cathepsin inhibitor 1 and PSI, both purchased from Calbiochem, UK.

Quantification of calpain activity

Analysis of calpain activity in total cell lysates was performed using a calpain activity assay kit from BIOvision, Inc. (Mountain View, CA) following the manufacturer's instructions. The calpain activity kit contains a fluorogenic peptide calpain substrate (Ac-LLY-AFC), lysis buffer, and reaction buffer. Briefly, 10^6 cells are lysed in lysis buffer for 20 minutes at 4°C. Clarified cell lysates are then incubated with substrate and reaction buffer for 1 hour at 37°C in the dark. Upon cleavage of substrate, the fluorogenic portion (AFC) of the calpain substrate releases fluorescence at a wavelength of 505 nm following excitation at 400 nm. Fluorescence emission was measured using a fluorescent plate reader (Chameleon multilabel detection platform; Hidex, Finland). For each sample, control reactions were performed in the presence of 5 μg recombinant calpastatin (Calbiochem, UK) to monitor any calpain-independent proteolysis of fluorogenic peptide. Values from control reactions were subtracted from total activity values to specifically determine calpain activity for each sample. Results are expressed as relative fluorescence units per microgram of protein lysate.

Immunocytochemistry

Cells cultured on poly-L-lysine (Sigma, UK)-coated coverslips overnight in complete culture medium were fixed for 20 minutes in 4% w/v paraformaldehyde/3% w/v sucrose in Dulbecco's PBS warmed to 37°C. They were then washed three times with PBS, permeabilised with 0.5% v/v Triton X-100 in PBS for 10 minutes and blocked with 5% w/v bovine serum albumin (BSA) in PBS for 45 minutes at room temperature. To block indiscrimate Fc-receptor binding, cells were incubated for 1 hour with a PBS solution containing 1:100 v/v anti-MoFc receptor (Research Diagnosis). Coverslips were washed three times with PBS and incubated for 1 hour at room temperature with 1:500 dilution of anti-vinculin or 1:100 dilution of anti-paxillin or anti-talin antibodies respectively in PBS containing 2% w/v BSA, followed by an incubation with a 1:200 dilution of Cy5- and Alexa Fluor 488-conjugated anti-mouse IgG, respectively in PBS. β_2 integrins were detected by incubating coverslips with a 1:100 dilution of the antibody in PBS containing 2% w/v BSA overnight at 4°C. After three washes with PBS, cells were

incubated in a dilution of 1:200 FITC-conjugated anti-rat antibody in PBS. Actin filaments were detected by incubation with a solution of 0.1 μg/ml Alexa Fluor 568-conjugated phalloidin in PBS for 1 hour at 37°C. Coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, UK) and visualised using a Zeiss LSM 510 Meta confocal laser scanning head attached to a Zeiss Axioplan 2 microscope. LSM 510 software was used to collect four sequential images from four separate optical sections in the *z* axis 0.2 μm apart. The same software was used to obtain merged confocal images. Quantification of fluorescent intensity in confocal micrographs was performed using Kinetic Imaging Software, UK.

Analysis of adhesion turnover

We used interference reflection microscopy (Curtis, 1964; Dunn and Jones, 2004) to visualise the adhesion-substratum interface of living cells. DCs in complete culture medium were plated on poly-L-lysine-coated glass coverslips and incubated overnight at 37°C in a 5% CO_2 atmosphere as previously described. Coverslips of untreated or treated DC with ALLM/ALLN (50 μM) or calpastatin peptide or scrambled calpastatin peptide (50 μM) were mounted onto viewing chambers in culture medium. Interference reflection micrographs were collected using a Zeiss Standard 18 microscope fitted with an incident light fluorescence attachment. Exciter and barrier filters were removed from the LP420 reflector and replaced with a narrow band-pass filter to isolate the 546 nm line of the mercury arc source. Coverslips with attached cells were observed using a Zeiss 63× Neofluar Antiflex oil-immersion objective, NA 1.25. Images were collected digitally using in-house software and were processed using Adobe Photoshop® version 7 to threshold the adhesion sites of the cells with the substratum.

The podosome turnover in DCs expressing eGFP constructs (see below) was performed by simultaneously visualising the GFP signal and adhesion-substratum interface using a Zeiss LSM Meta confocal scanning head as described above, using the 488 nm line of an Argon laser and a 470-500 nm band-pass filter to detect the eGFP signal and a 505 nm long-pass filter to detect the interference reflection signal. To analyse the persistence of adhesion sites, ten images taken 30 seconds apart were overlapped using the 'difference' function in Adobe Photoshop. We thus obtained a composite image with ten relevant grey levels. The areas of light-grey colour pixels represent dynamic adhesions whereas areas of dark-grey and black pixels represent increasingly stable adhesions during the selected time course of measurement. Using the 'histogram' function of Adobe Photoshop, we could quantify the percentage of pixels per image corresponding to each grey level, which allowed us to calculate a turnover index by dividing the percentage of pixels present in frames 1 to 5 by the percentage of pixels present in frames 6 to 10 (Holt et al., personal communication). The Student's *t*-test test was used to assess the statistical significance of experimental results (*P<0.05).

Cell migration speed

DCs were plated overnight on poly-L-lysine-coated glass coverslips as previously described and mounted onto viewing chambers in cell culture medium. Phase-contrast micrographs (magnification, 10× lens) taken 5 minutes apart for 4 hours were collected using a Zeiss Axiovert 35 connected to a Hamamatsu digital camera and recorded digitally using AQM software (Kinetic Imaging, Nottingham, UK). Cell migration was tracked using Kinetic Imaging motion analysis software and speeds calculated from displacements of the cell nuclei.

Cell spreading assay

Suspensions of DCs in complete culture medium in the presence or absence of calpain inhibitors were plated on poly-L-lysine-coated Petri dishes at 37°C in a 5% CO_2 atmosphere. Phase-contrast micrographs (magnification, 10× lens) were taken every 5 minutes for 4 hours and collected using the same set-up described above. Cell adhesion areas with respect to time were measured using motion analysis software as before.

Cell adhesion assay

DCs were harvested, washed twice with RPMI and plated in RPMI onto poly-L-lysine-coated 96-well plates (50,000 cells/well) and incubated for 30 minutes at 37 C in a 5% CO_2 atmosphere. The assay was terminated by aspiration of the medium, followed by three washes with PBS to remove unbound or loosely bound cells. Substratum-bound cells were fixed with 4% w/v paraformaldehyde/3% w/v sucrose for 20 minutes and then stained with a 0.1% solution of Crystal Violet for 2 hours at room temperature. Excess crystal violet was washed off with distilled water and the plate was air-dried. Cells were lysed with a 1% SDS solution and optical density/well was measured at 580 nm in a Multiscan MCC/340 MkII spectrophotometer. We found 95-100% DCs plated under the conditions described were securely bound onto the substratum after 4 hours, so we set this value as 100% adhesion of DCs for comparison with data collected for treated cell suspensions.

Cell transmigration assay

A confluent monolayer of SVEC 4-10 cells was generated by plating 3×10^4 cells on endothelial cell attachment factor (TCS CellWork Ltd)-coated 13-mm-diameter coverslips in 24-well plates overnight. SVEC 4-10 cells were activated to induce maximal expression of cell adhesion molecules by incubation with 250 U/ml TNF-α (R&D Systems) for 6 hours. DCs were fluorescently labelled by incubation in CFSE (Molecular Probes, UK) and 25×10^3 cells seeded per well in 0.5 ml RPMI. After 1 hour, co-cultures of DCs and SVEC were washed once with PBS at 37ºC and fixed for 20 minutes in 4% w/v paraformaldehyde/3% w/v sucrose in PBS at 37°C. Coverslips were stained with Alexa Fluor 568 phalloidin to detect F-actin and mounted onto slides. Three sequential confocal optical sections were taken at the top, centre and bottom of the SVEC monolayer of randomly chosen fields. We scored the percentage of DCs per coverslip found on either the surface of the monolayer, spanning the monolayer, or having fully crossed the monolayer for 25 cells per coverslip and four coverslips per experiment.

Western blotting

Calpain inhibitor treated or control DCs under the required experimental conditions were lysed in RIPA lysis buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium molybdate, 20 mM phenylphosphate with protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, 50 mM NaF and 1 mM Sodium orthovanadate]. 20 μg total cell lysate protein was loaded per lane in a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and subjected to electrophoresis. Proteins were blotted onto PVDF membranes using a Bio-Rad Mini protein II transfer apparatus. Blots were blocked with 5% dried milk solution diluted in TBS-T (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), incubated with indicated antibody and signal detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection system. Blots were stripped for 30 minutes with 2% SDS and 0.7% β-mercaptoethanol for 1 hour at 50°C and reprobed for β-actin to confirm the total amount of protein loaded per lane.

Infections of DC using lentiviral vectors

Lentiviral vector stocks were produced in 293T cells by co-transfecting the transfer vector pHR′SINcPPT-SFFV-eGFP-(SEW) or SFFV-eGFP-WASp (SEWW), the envelope plasmid pMD.G, and the packaging plasmid pCMVR8.91, as previously described (Zufferey et al., 1997). 1.5×10^7 cells were seeded onto 150 cm^2 flasks and transfected with 10 μg DNA envelope, 30 μg DNA packaging and 40 μg DNA transfer vector by precomplexing with 0.125 μM PEI (22 kDa) for 15 minutes at room temperature in OptiMEM. After 4 hours at 37°C the medium was replaced with fresh DMEM 10% FCS and virus were harvested 48 and 72 hours post transfection. After filtering through a 0.45 μm-pore-size filter, the virus suspension was concentrated by centrifugation at 50,000 ***g*** for 2 hours at 4°C. The resulting pellet was resuspended in RPMI (Sigma, UK) and stored at –80°C until use. The desired number of DCs were plated in complete culture medium as described above using phenol-free RPMI (Sigma, UK) and lentivirus-containing supernatant was added to the cells at an MOI of 100 and incubated for 24 hours. Medium was replaced with complete DC culture medium without phenol-free RPMI after 24 hours and cells were cultured for a further 48 hours to allow maximal expression of lentiviral vectors before being used in experiments.

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