The role of cathepsin X in the migration and invasiveness of T lymphocytes

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Summary

Cathepsin X is a lysosomal cysteine protease exhibiting carboxypeptidase activity. Its expression is high in the cells of immune system and its function has been related to the processes of inflammatory and immune responses. It regulates processes such as adhesion, T lymphocyte activation and phagocytosis through its interaction with β2 integrins. To investigate the role of cathepsin X in the migration of T lymphocytes, Jurkat T lymphocytes were stably transfected with a pcDNA3 expression vector containing cathepsin X cDNA. The cathepsin-X-overexpressing T lymphocytes exhibited polarised migration-associated morphology, enhanced migration on 2D and 3D models using intercellular adhesion molecule 1 (ICAM1)- and Matrigel-coated surfaces, and increased homotypic aggregation. The increased invasiveness of cathepsin-X-overexpressing cells does not involve proteolytic degradation of extracellular matrix. Confocal microscopy showed that the active mature form of cathepsin X was colocalised in migrating cells together with lymphocyte-function-associated antigen 1 (LFA-1). The colocalisation was particularly evident at the trailing edge protrusion, the uropod, that has an important role in T lymphocyte migration and cell-cell interactions. We propose that cathepsin X causes cytoskeletal rearrangements and stimulates migration of T lymphocytes by modulating the activity of the β2 integrin receptor LFA-1.

Supplementary material available online at
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Key words: Cathepsin X, T lymphocytes, Integrins, Migration, Invasion

Introduction

Cathepsin X (also known as cathepsin Z or CATZ) belongs to the C1 family of lysosomal cysteine proteases. Its gene (*CTSZ*) (Santamaria et al., 1998; Nagler et al., 1998), structure (Gunčar et al., 2000; Sivaraman et al., 2000) and activity properties (Nagler et al., 1999; Klemenčič et al., 2000) show several unique features that distinguish it clearly from other human cysteine proteases. It has a very short pro-region that shows no similarity to those of other cathepsins (Sivaraman et al., 2000) and a three-residue insertion motif that forms a characteristic 'mini loop' (Gunčar et al., 2000). Cathepsin X exhibits mono- and di-peptidase activity at its C-terminus (Gunčar et al., 2000) and, in contrast to cathepsin B, does not act as an endopeptidase (Menard et al., 2001). Contrary to the first reports (Santamaria et al., 1998) cathepsin X is not widely expressed in cells and tissues, but is restricted to the cells of the immune system, predominantly monocytes, macrophages and dendritic cells (Kos et al., 2005). Higher levels of cathepsin X were also found in tumour and immune cells of prostate (Nagler et al., 2004) and gastric (Buhling et al., 2004) carcinomas and in macrophages of gastric mucosa, especially after infection by *Helicobacter pylori* (Krueger et al., 2005). Recently it has been shown that cathepsin X is abundantly expressed in mouse brain cells, in particular glial cells, and that its concentration increased with age. Its upregulation has also been detected in the brain of patients with Alzheimer disease (Wendt et al., 2007).

The integrin-binding motifs, present in the pro-form (Arg-Gly-Asp: RGD) and in the mature form (Glu-Cys-Asp: ECD) of cathepsin X (Santamaria et al., 1998) suggest a role in integrin-mediated signal transduction. Moreover, cathepsin X binds cell-surface heparan sulfate proteoglycans (Nascimento et al., 2005), which are also involved in integrin regulation (Beauvais et al., 2004). Integrins are a family of glycosylated, heterodimeric transmembrane-adhesion receptors that consist of noncovalently bound α- and β-subunits. Integrins may bind to counter-receptors on other cells or mediate interactions with components of the extracellular matrix (ECM) (Hynes, 2002). Following adhesion and clustering, integrins recruit various cytoskeletal and cytoplasmic proteins, and anchor the complexes to the actin cytoskeleton. This ultimately leads to the local remodelling of actin and the formation of specialised adhesive structures, called focal adhesions (van der Flier and Sonnenberg, 2001). In addition to forming a structural link between the ECM and the actin cytoskeleton, focal adhesions are important sites of signal transduction, connecting integrin-mediated adhesion with the pathways that control various cellular processes, such as migration, proliferation and differentiation (Lauffenburger and Horwitz, 1996; Longhurst and Jennings, 1998).

The pro-form of cathepsin X interacts with αvβ3 integrin through the RGD motif in lamellipodia of human umbilical vein endothelial cells (HUVECs) (Lechner et al., 2006). A strong colocalisation with the β3-integrin subunit was also confirmed in pro-monocytic U-937 cells (Obermajer et al., 2006a). However, the active form of cathepsin X colocalised predominantly with the β3-integrin subunit in various cells of monocytes and macrophage lineage. Active cathepsin X has been shown to regulate β2-integrin-dependent adhesion, phagocytosis and activation of T lymphocytes (Obermajer et al., 2006a) by interacting with lymphocyte function-associated antigen 1 (ITAL, also known as and hereafter referred to as LFA-1) and macrophage

antigen 1 (Mac-1; also known as integrin beta 2 or ITGB2) (Obermajer et al., 2008).

The role of β2-integrin receptors is important also in other T lymphocyte functions, such as migration and invasion across the endothelium and tissues (Van Andrian and Mackay, 2000). To investigate the potential role of cathepsin X in the regulation of these processes we overexpressed cathepsin X in T lymphocytes (which normally express low levels of the enzyme). We showed that cathepsin X is involved in cytoskeleton-dependent morphological changes of T lymphocytes. Moreover, in 2D and 3D models that mimicked the extracellular matrix, we demonstrated that cathepsin X enhances T lymphocyte migration, invasiveness and homotypic aggregation. We found that cathepsin X colocalised with LFA-1, which has previously been shown to induce homotypic aggregation and cytoskeletal changes in T lymphocytes.

Results

Overexpression of cathepsin X in stably transfected T lymphocytes

Stable transfectants of Jurkat T lymphocytes that overexpress cathepsin X were generated and selected from the total population by limiting-dilution to a single cell per well. Several resistant clones were obtained and the expression of cathepsin X was quantified by ELISA (Fig. 1A). In cytosols of wild-type cells and overexpressing cells containing the clone with the highest productivity (clone 17), the levels of cathepsin X were 1.5 ng/mg (ng of cathepsin X/mg of total cell protein) and 35.5 ng/mg, respectively ($P<0.001$) (Fig. 1B). The increase of cathepsin X activity in overexpressing cells (Fig. 1C) was visualised by the activity-dependent probe DCG-04, which targets a broad set of cysteine proteases including cathepsins B, S, L, H, C and X (Lennon-Dumenil et al., 2002). The position of the bands that correspond to individual cathepsins was the same as reported in previous studies (Fig. 1C) (Lennon-Dumenil et al., 2002; Verhelst and Bogyo, 2005; Sadaghiani et al., 2007). Cathepsin X activity was 2.3-fold higher in overexpressing than in wild-type cells when comparing the medium density of the bands (Fig. 1C). The identity of the band was confirmed as being cathepsin X (by using cathepsin-X-specific 2F12 mAb) (Fig. 1C). Expression of cathepsin X on the cell surface was determined by flow cytometry using Alexa-Fluor-488-labelled 2F12 mAb. It was evident that both, wild-type and cathepsin-X-overexpressing Jurkat T lymphocytes express low levels of cathepsin X on cell surface (Fig. 1D). Quantitative real-time PCR (qPCR) analysis was also performed to determine the relative cathepsin X mRNA expression levels in wild-type and stably transfected Jurkat T lymphocytes. The expression level of cathepsin X in stably transfected Jurkat T lymphocytes (clone 17) was up 71 times (supplementary material Fig. S1E). The data were normalised to two endogenous controls, hypoxanthine-guanine phosphoribosyl transferase (HPRT) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ).

Overexpression of cathepsin X correlates with the increasing homotypic aggregation of T lymphocytes

Jurkat T lymphocytes were incubated in complete RPMI medium for 24 hours and observed for cell aggregation. Only some of the wild-type Jurkat T lymphocytes were prone to aggregation, forming small cell clusters (Fig. 2A). By contrast, extensive homotypic aggregation was observed for the majority of cathepsin-X-overexpressing cells (Fig. 2B). Homotypic aggregation of cathepsin-X-overexpressing cells was less extensive in the presence of 0.25 mM EDTA, which may interfere with the interaction between LFA-1 and ICAM1 (Labadia et al., 1998). Both, 2F12 mAb and the cell-permeable epoxysuccinyl-based cathepsin-X-specific inhibitor AMS36 (Sadaghiani et al., 2007) reduced homotypic aggregation of cathepsin-X-overexpressing Jurkat T lymphocytes.

Cathepsin X induces an orientated movement resulting in homotypic aggregation and the formation of extended uropods in T lymphocytes migrating on ICAM1

To study the involvement of LFA-1 in the migration of Jurkat T lymphocytes we followed their migration on an ICAM1-coated

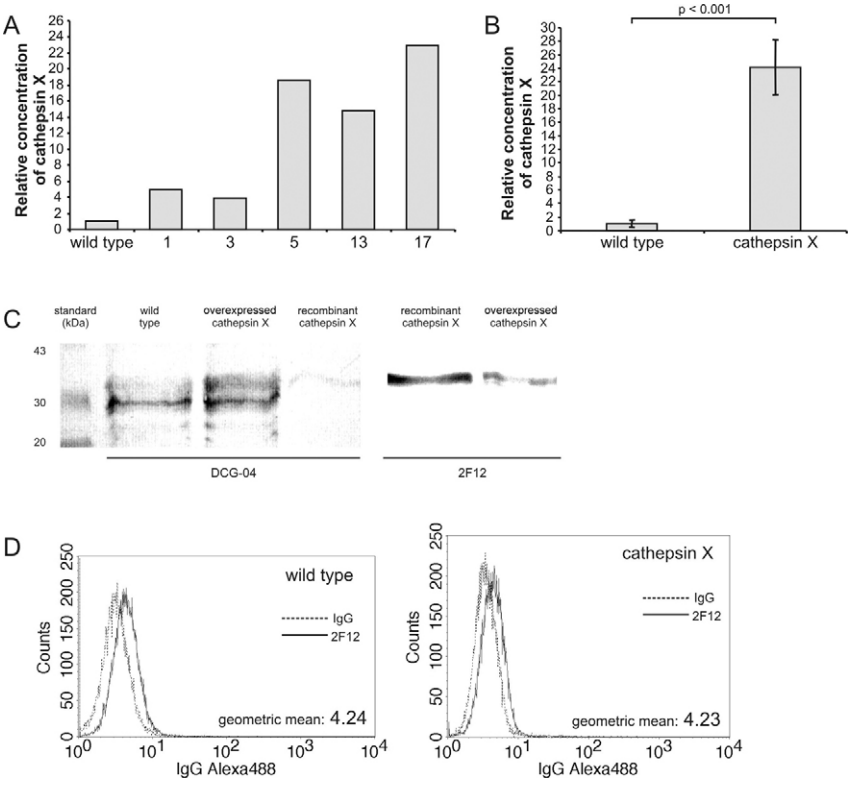

Fig. 1. Protein, activity and mRNA levels of cathepsin X in stably transfected Jurkat T lymphocytes (A) Protein levels of cathepsin X in the cytosol of resistant clones as determined by ELISA. The highest cathepsin X productivity was observed in clone 17, which was selected for further studies. (B) The molar level of cathepsin X in stably transfected Jurkat T lymphocytes with the highest productivity was 24-fold higher compared with wild-type cells. The mean levels obtained for wild-type Jurkat T lymphocytes (1.5 ng of cathepsin X per mg of total cell proteins) have been normalised to a relative concentration of 1.0. (C) Cathepsin X activity was determined by DCG-04 active-site labelling and the identity of cathepsin X in the cytosol was confirmed by immunoblotting. Activity of cathepsin X was increased in cathepsin-X-overexpressing T lymphocytes compared with wild-type cells. (D) Cathepsin X expression on the cell surface was unchanged after cathepsin X overexpression, as measured by flow cytometry. The geometric mean was 4.24 for wild-type and 4.23 for cathepsin-X-overexpressing T lymphocytes.

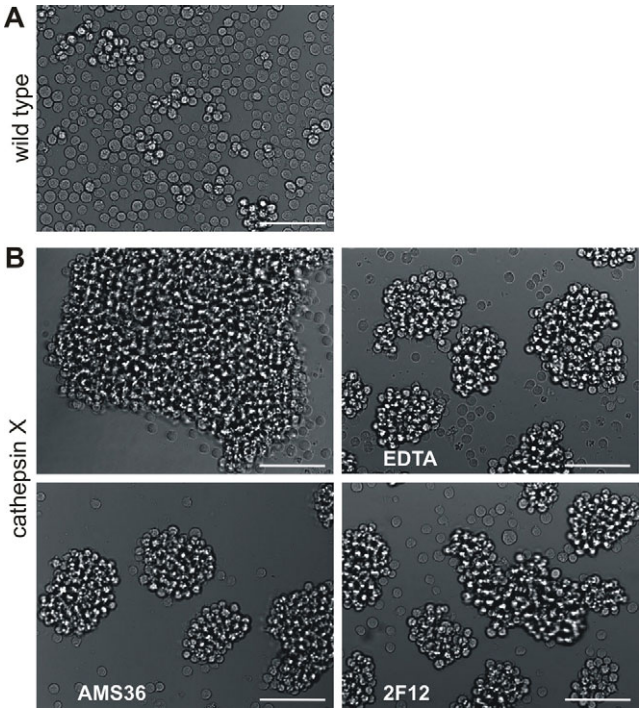

Fig. 2. Homotypic cell aggregation of cathepsin-X-overexpressing T lymphocytes. Jurkat T lymphocytes were incubated for 24 hours in complete RPMI. (A) Wild-type Jurkat T lymphocytes show weak aggregation (B) Upregulation of cathepsin X in Jurkat T lymphocytes resulted in formation of large cell aggregates. Addition of EDTA, 2F12 cathepsin-X-neutralising mAb or cathepsin-X-specific inhibitor AMS36 reduced homotypic aggregation of cathepsin-X-overexpressing Jurkat T lymphocytes. Scale bars, 100 μm.

surface (Stanley and Hogg, 1998). Under time-lapse microscopy cathepsin-X-overexpressing cells started immediately to move towards each other, resulting in the formation of aggregates within 2 hours. However, the migration of wild-type cells was less marked and aggregation was not oriented (Movies 1, 2 in supplementary material). PMA, which is known to regulate the interaction between LFA-1 and the cytoskeleton, as well as integrin clustering on the cell surface (Liliental and Chang, 1998), was used for the activation of cells. It induced the formation of long uropods attached either to ICAM1-coated surface or to other cathepsin-X-overexpressing cells (Fig. 3A). By contrast, PMA-activated wild-type cells show no phenotypic changes after 24 hours of incubation on ICAM1.

Overexpression of cathepsin X promotes migration of T lymphocytes

The migration of Jurkat T lymphocytes was followed on polycarbonate membranes coated with ICAM1. On uncoated membrane the migration of cathepsin-X-overexpressing cells increased 4.4-fold compared with than that of wild-type cells ($P<0.001$) (Fig. 4A). 2F12, the cathepsin-X-neutralising mAb reduced the migration of cathepsin-X-overexpressing Jurkat T lymphocytes by 33% and cell-permeable cathepsin-X-specific inhibitor AMS36 by 49%. Migration of cathepsin-X-overexpressing Jurkat T lymphocytes on ICAM1-coated membranes was increased 1.9-fold compared with that of the wild-type cells ($P<0.001$) (Fig. 4B). In this case, 2F12 mAb reduced the migration of cathepsin-X-overexpressing Jurkat T lymphocytes by 10% and AMS36 by 21%.

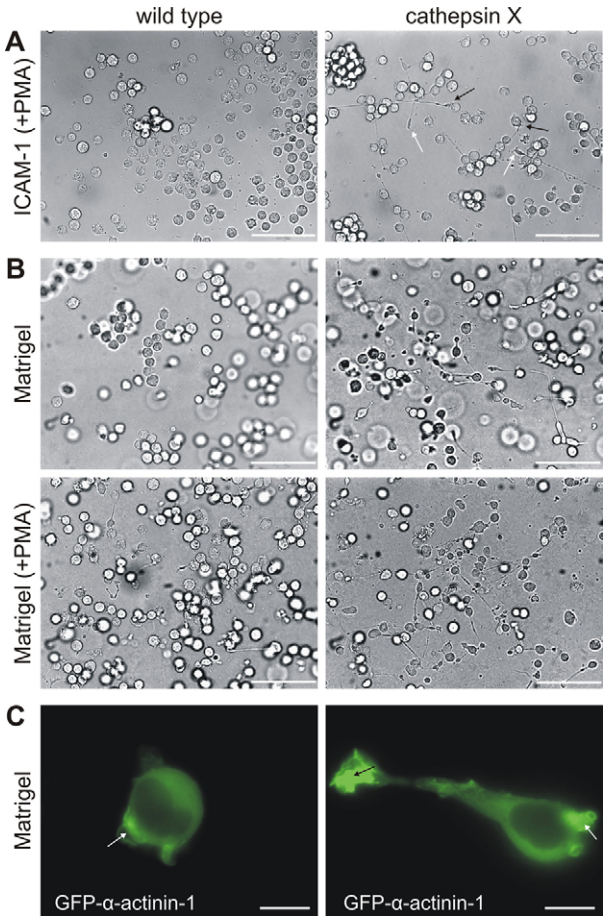

Fig. 3. Morphological changes in T lymphocytes migrating on ICAM1 (upper panel) and Matrigel (lower panel). (A) Cells were activated with PMA and incubated for 24 hours on immobilised ICAM1. Wild-type Jurkat T lymphocytes show no evident phenotypic changes whereas overexpression of cathepsin X in Jurkat T lymphocytes resulted in the development of extended uropods. White arrows indicate the uropods attached to ICAM1-coated surface and black arrows the uropods attached to other cells. (B) Wild-type Jurkat T lymphocytes remained in a spherical non-migratory state after 48 hours of incubation on Matrigel. Activation with PMA resulted in the development of cytoskeletal rearrangements in a small proportion of the wild-type cells. By contrast, in transfected cells cathepsin X induced the formation of a polarised phenotype with long uropods, independently of the activation with PMA. (C) In cathepsin-X-overexpressing cells the accumulation of GFP–α-actinin-1 at the lamellipod (white arrows) is more evident than in wild-type cells. GFP–α-actinin-1 is concentrated also at the adhesive tip of the uropod (black arrow) formed only in cathepsin-X-overexpressing cells. Scale bars, 100 μm (A,B) and 10 μm (C).

Localisation of active LFA-1 and cathepsin X in the migrating T lymphocytes

The distribution of active LFA-1 in Jurkat T lymphocytes was determined by Alexa-Fluor-488-conjugated mAb 24, which is specific for active LFA-1. After 15 minutes of incubation on ICAM1 we observed morphological changes in the majority of cathepsin-X-overexpressing cells, resulting in a polarised phenotype with lamellipodia, mid-cell region and uropod, whereas no obvious changes in shape were detected in most of the wild-type cells. In spherical cells which did not undergo evident morphological changes, active LFA-1 was localised predominantly at the perimembrane region (Fig. 5A). In polarised migrating cathepsin-X-overexpressing cells active LFA-1 was found also at the uropod

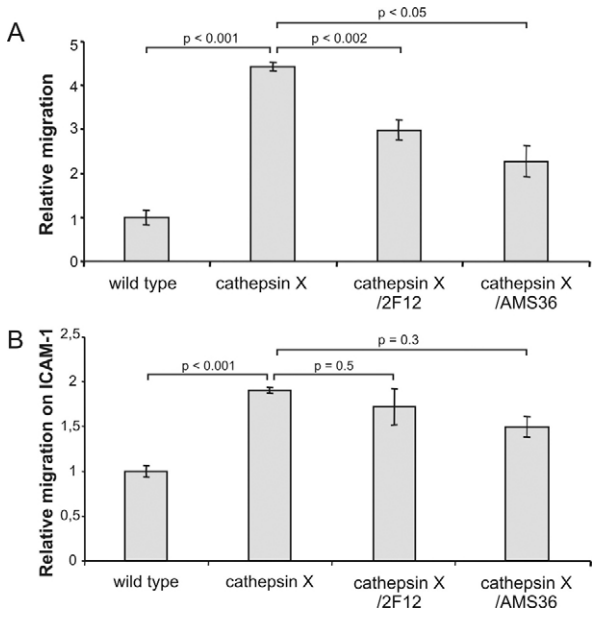

Fig. 4. Effect of cathepsin X on T lymphocyte migration. Data are expressed as the mean ± s.d. of triplicate wells. The percentage of migration obtained for wild-type cells was normalised to 1. (A) Cathepsin-X-overexpressing Jurkat T lymphocytes migration on uncoated polycarbonate membranes. (B) Cathepsin-X-overexpressing Jurkat T lymphocyte migration on ICAM1 coated membranes. 2F12, cathepsin-X-neutralising mAb (1 μM); AMS36, epoxysuccinyl-based specific inhibitor of cathepsin X (2 μM).

(Fig. 5B). Confocal imaging along the *z* axis demonstrated that the distribution of active LFA-1 was substantially increased in two cellular locations – in the mid-cell region that is in contact with the ICAM1-coated surface and in the protruding uropod (Fig. 5C). The colocalisation of mAb 2F12, which is specific for active cathepsin X, and the common lysosomal marker protein LAMP2 (Kokkonen et al., 2004) shows that a part of active cathepsin X is released from the lysosomes, and localised at the perimembrane and uropod region. The majority of lysosomes is located in the perinuclear region, but not in the uropod (Fig. 5D).

Colocalisation of cathepsin X and LFA-1 in T lymphocytes that migrate on ICAM1 and 3D Matrigel

To evaluate possible role of cathepsin X in the LFA-1-mediated cytoskeletal rearrangement and migration, we found that, using specific antibodies, mature cathepsin X and LFA-1 colocalised in Jurkat T lymphocytes . On ICAM1 active cathepsin X colocalised with LFA-1 in both wild-type and cathepsin-X-overexpressing Jurkat T lymphocytes. In wild-type cells, which were manly in a spherical, non-migratory form, colocalisation was observed at the perimembrane region, whereas in polarised cathepsin-X-overexpressing cells, LFA-1 and cathepsin X colocalised predominantly in the uropod (Fig. 6A,B). A similar colocalisation profile was obtained when cells were grown on 3D Matrigel (Fig. 6C,D).

Cathepsin X causes uropod formation in T lymphocytes on Matrigel

Jurkat T lymphocytes were tracked in the 3D Matrigel migration model by video microscopy after a 24-hour incubation. The migration of cathepsin-X-overexpressing cells was dynamic, accompanied by extensive changes in shape and the formation of

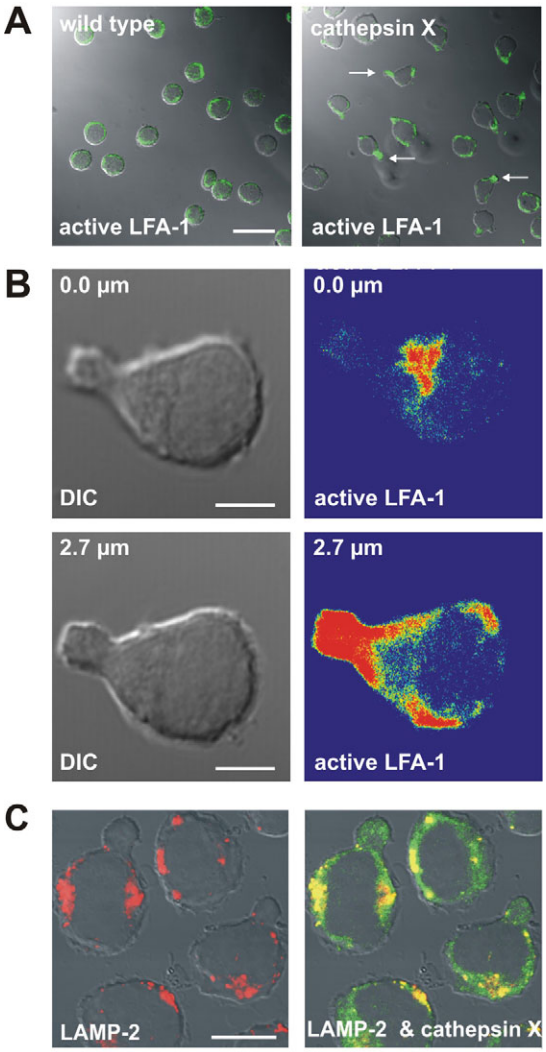

Fig. 5. Localisation of active LFA-1 and cathepsin X in the migrating T lymphocytes. Jurkat T lymphocytes were seeded on ICAM1 pre-coated slides and allowed to migrate for 15 minutes before labelling. Active LFA-1 was detected with Alexa_fluor-488-conjugated specific antibody mAb 24. (A) Wild-type Jurkat T lymphocytes. Active LFA-1 is localised manly in the perimembranous region of the spherical non-migratory state cells. Cathepsin-X-overexpressing Jurkat T lymphocytes showing polarised phenotype. Active LFA-1 is localised predominantly at the uropod region (white arrows). (B) Colocalisation of active LFA-1 and cathepsin X in upregulated migratory cells along the *z* axis. Active LFA-1 is localised at the mid-cell region, which is in contact with ICAM1-coated surface (0.0 μm) and at the uropod projecting above the surface (2.7 μm). (C) Colocalisation of active cathepsin X (green; Alexa-Fluor-488) and the lysosomal marker protein LAMP2 (red; Alexa-Fluor-546) in cathepsin-X-overexpressing T lymphocytes. Cathepsin X is colocalised in the lysosomes (yellow), it is also present in perimembrane region and uropod (green). Scale bars, 50 μm (A), 5 μm (B), 10 μm (C).

long uropods, whereas the migration of wild-type cells was relatively stationary and only a small fraction of the cells showed the formation of a polarised phenotype (Movies 3, 4 in supplementary material). Fig. 3B shows cells after 48 hours of migration on Matrigel. Wild-type cells show no shape changes, whereas the majority of cathepsin-X-overexpressing Jurkat T lymphocytes exhibit a polarised migratory phenotype. When the cells were activated with PMA, a limited number of uropods formed in wild-type cells, however, the effect was less extensive than in cathepsin-

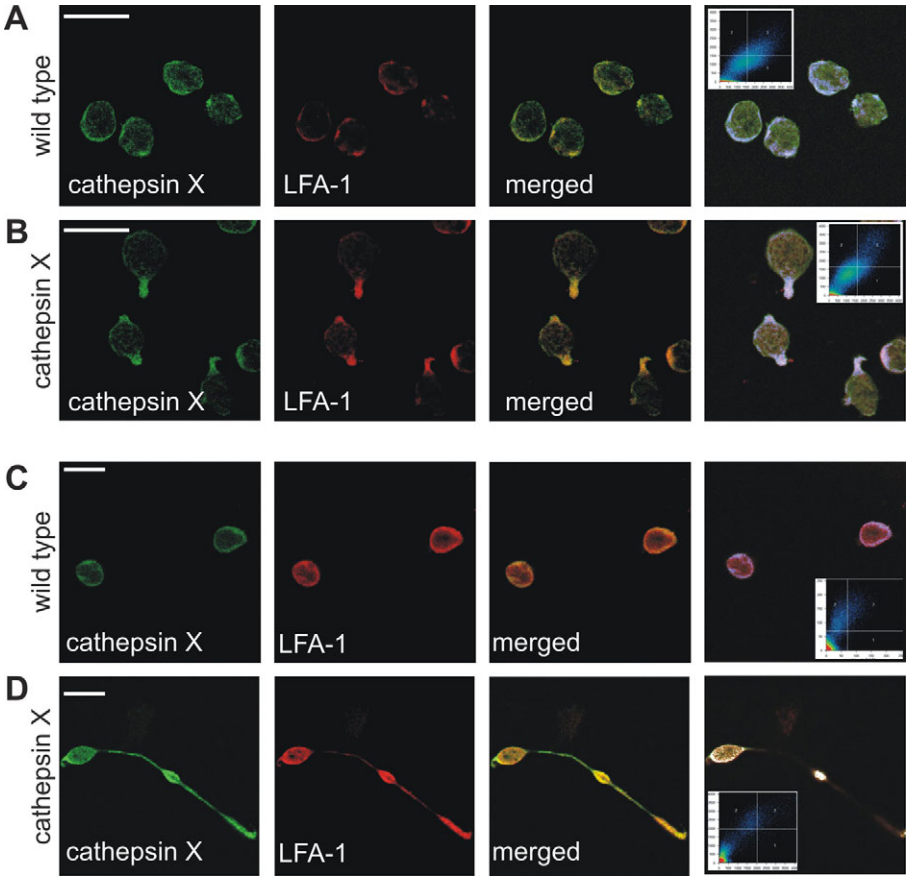

Fig. 6. (A-D) Colocalisation of cathepsin X (green; Alexa-Fluor-488) and LFA-1 (red; Alexa-Fluor-633) in Jurkat T lymphocytes migrating on ICAM1 (A,B) and Matrigel (C,D). Cells were seeded on pre-coated slides and allowed to migrate for 15 minutes (ICAM1) or 48 hours (Matrigel) before labelling. (A) In wild-type Jurkat T lymphocytes no morphological changes were observed, cathepsin X and LFA-1 are colocalised mainly in the perimembranous region. (B) In cathepsin-X-overexpressing Jurkat T lymphocytes with a polarised migratory phenotype cathepsin X and LFA-1 are colocalised predominantly at the uropod. (C,D) A similar colocalisation profile was obtained in 3D Matrigel. In cathepsin-X-overexpressing cells (D) with a polarised migratory phenotype cathepsin X and LFA-1 are colocalised at the perimembrane region and at the uropod. Fluorescent dyes were imaged sequentially in all colocalisation experiments in a frame-interlace mode to eliminate cross talk between the channels. The threshold level for this display was set to 90, which corresponds to two-thirds of the maximal brightness level. Pixels above the threshold in both channels (blue to white colour) and the contour plot are shown for images that demonstrate colocalisation. Scale bars, 20 μm.

X-overexpressing cells. The cytoskeletal rearrangements were followed by the GFP-linked cytoskeletal protein α-actinin-1 (Fig. 3C). In wild-type cells, GFP–α-actinin-1 was distributed throughout the cells, being more concentrated in the forming lamellipodium. In cathepsin-X-overexpressing cells the accumulation of GFP–α-actinin-1 at the lamellipodium is more evident. Moreover, GFP-α-actinin-1 is localised in the uropod, particularly in its adhesive tip.

Upregulation of cathepsin X promotes the invasion of T lymphocytes into Matrigel

The effect of cathepsin X on Jurkat T lymphocyte invasion was evaluated using Matrigel-coated polycarbonate membranes. By analogy to the migration assay, cathepsin X increased the invasion of Jurkat T lymphocytes. The invasiveness of cathepsin-X-overexpressing Jurkat T lymphocytes was 8.3-fold higher than that of wild-type cells ($P<0.001$). 2F12 neutralising mAb reduced the invasiveness by 29% and cell-permeable cathepsin-X-specific inhibitor AMS36 by 33% (Fig. 7).

T lymphocyte migration through Matrigel does not depend on proteolytic degradation of the ECM

To exclude the involvement of cathepsin X in proteolytic degradation of ECM, we tested cathepsin-X-overexpressing Jurkat T lymphocytes for degradation of collagen IV, the major ECM component, during migration in Matrigel (Paulsson, 1992). Cells were seeded on the top of a 3D Matrigel mixed with quenched fluorescent substrate DQ-collagen-IV and observed the development of fluorescent degradation products. The highly invasive MCF-10A neoT cells exhibited extensive extracellular and intracellular degradation (green fluorescence) of DQ-collagen-IV (Fig. 8A). Wild-type Jurkat T lymphocytes showed no invasive morphology and no fluorescent collagenolytic products (Fig. 8B). Cathepsin-X-overexpressing Jurkat T lymphocytes adopted a characteristic migrating phenotype with long uropods and as in wild-type cells, the Matrigel invasion process was not accompanied by proteolytic degradation of collagen (Fig. 8C).

Discussion

Active cell migration is essential for physiological tissue development and homeostasis. This process is particularly important for the T lymphocyte immunosurveillance function and inflammatory response and involves interactions of T lymphocytes with endothelium and extracellular matrix (Friedl and Brocker, 2000), which are mediated mostly by integrins. Inside the cell integrins modulate the cytoskeletal organisation (Lauffenburger and Horwitz, 1996) that result in cell polarisation and the changes in cell shape that allow the conversion of cytoskeletal forces into net displacement of the cell (Sanchez-Madrid and del Pozo, 1999).

Cathepsin X interacts with integrins in two ways. First its pro-form binds by the RGD motif to the active extracellular integrin ligand binding domain, competing with the binding of the components of ECM and triggering outside-in signalling. Second, active mature cathepsin X colocalises with and activates β2 integrin receptors Mac-1 and LFA-1, which are abundantly expressed on leukocytes (Obermajer et al., 2006a). In this way it stimulates Mac-1 receptor-dependent adhesion and modulates the proliferation of lymphocytes. As shown recently the activation of Mac-1 receptor inhibits antigen presentation and downregulate T lymphocyte

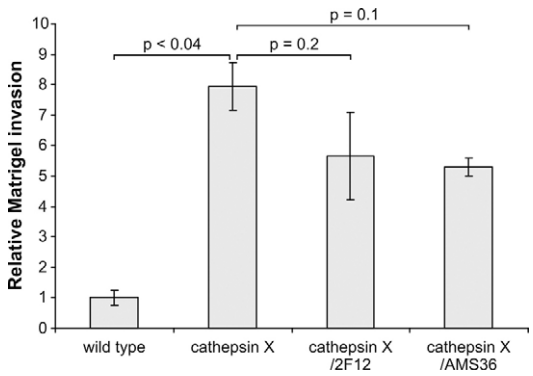

Fig. 7. Effect of cathepsin X on T lymphocyte Matrigel invasion. The percentage of invasion obtained for wild-type cells was normalised to 1. Cathepsin X transfection increased Jurkat T lymphocyte invasion through Matrigel 8.3-fold compared with wild-type cells. The invasion of cathepsin-X-overexpressing Jurkat T lymphocytes was inhibited by 29% by 2F12 anti-cathepsin-X-neutralising mAb and by 33% by cathepsin-X-specific inhibitor AMS36 (Fig. 7). Data are expressed the mean ± s.d. of triplicate wells.

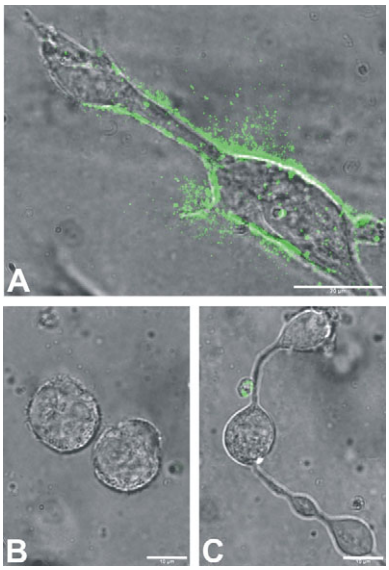

Fig. 8. Degradation of DQ-collagen-IV by living cells that migrate through Matrigel. Cells were incubated for 48 hours on Matrigel mixed with DQ-collagen-IV. (A) Degradation of DQ-collagen-IV by living MCF-10A neoT cells; green fluorescent degradation products of DQ-collagen-IV are present extracellularly and intracellularly. (B) Wild-type Jurkat T lymphocytes show no morphology changes and no fluorescent degradation products. (C) Cathepsin-X-overexpressing Jurkat T lymphocytes show a characteristic migratory phenotype and, similar to wild-type Jurkat T lymphocytes, no collagen degradation is observed. Scale bars, 20 μm (A) and 10 μm (B,C).

activation (Varga et al., 2007), whereas the activation of LFA-1 promotes it. By blocking cathepsin X function against a particular integrin receptor we were able to promote the activation and enhance the proliferation of T lymphocytes (Obermajer et al., 2008).

In previous studies the effect of cathepsin X on integrin receptors has been studied by using protease inhibitors and specific neutralising monoclonal antibodies. Although the effects on integrin action were significant, the lack of specificity in the case of inhibitors and the weak internalisation of antibodies limit their application. To overcome these problems we stably transfected Jurkat T lymphocytes with the pcDNA3 expression vector containing cathepsin X cDNA. The expression of cathepsin X increased up to 20 times as confirmed by ELISA and qPCR. Also, the activity of cathepsin X was increased significantly in transfected cells, as determined by the DCG-04 active-site probe, specific for cysteine proteases (Greenbaum et al., 2000). The location of overexpressed cathepsin X was predominantly intracellular, the surface levels in both wild-type and cathepsin-X-overexpressing cells were low, as determined by flow cytometry. The same observation was shown for another T lymphocyte cell line, Mo-T, whereas in the pro-monocytic U-937 cells the surface fraction of cathepsin X was greater (Obermajer et al., 2008).

Wild-type Jurkat T lymphocytes express low levels of cathepsin X, whereas the expression of LFA-1 is considerable. LFA-1 activation results in either clustering of integrins on the cell surface (van Kooyk et al., 1994), or increased affinity for ligand binding induced by a conformational change (Hynes, 1992). Ligation of cell-surface receptors, such as TCR or chemokine receptors, coupled with phorbol ester (PMA) activation promotes LFA-1 clustering and cell adhesion (Stewart et al., 1998). Addition of the divalent cations Mn^{2+} or Mg^{2+} (Dransfield et al., 1992), or of activating antibodies (Kelleher et al., 1995) that bind to the extracellular domain of LFA-1 causes conformational changes that activate LFA-1. In vitro, outside-in activation increases the affinity of LFA-1, whereas inside-out activation induces an increase in its lateral mobility and clustering (Stewart et al., 1996; Hogg et al., 2003).

LFA-1 binds to its major counter receptor, the intercellular adhesion molecule 1 (ICAM1) (Marlin and Springer, 1987) and, with lower affinity, also to ICAM2 (Staunton et al., 1989) and ICAM3 (de Fougerolles and Springer, 1992). ICAM1 is widespread on most leukocytes and endothelial cells (Staunton et al., 1990; Campanero et al., 1993). T lymphocyte crawling across the endothelium is mediated by LFA-1 interacting with ICAM1 (Dustin et al., 1992). LFA-1 and ICAM1 are also known to be key molecules participating in cell aggregation (Petruzzelli et al., 1998). In this study, we found that overexpression of cathepsin X induces the homotypic aggregation of Jurkat T lymphocytes, presumably mediated by the LFA-1–ICAM1 pathway. Similar to EDTA, which has been suggested to interfere with LFA-1–ICAM binding, the presence of 2F12 mAb and the cathepsin-X-specific inhibitor AMS36 significantly reduced the size of the aggregates.

In addition to its adhesive properties, LFA-1 can act as a true signalling receptor, causing F-actin reorganisation that leads to cytoskeletal changes of the cell (Porter et al., 2002) and a switch from spherical to polarised (Coates et al., 1992). T lymphocyte migration is initiated by attachment to and crawling across the endothelium (Springer, 1994). After successful transmigration, moving T lymphocytes are confronted with a 3D network of multivalent ECM ligands, consisting of collagen, fibronectin, hyaluronan and other components (Ratner et al., 1992). T lymphocytes are known to form short-lived interactions with collagen fibres in the absence of ECM remodeling (Wolf et al., 2003), whereas the migration of large spindle-shaped cells, i.e. fibroblasts, endothelial cells and many tumour cells, is associated with extensive matrix degradation. The lysosomal cysteine proteases cathepsin B and L are capable of degrading the proteins of the basement membrane and ECM, such as laminin, fibronectin and collagen IV, thus facilitating tumour cell migration, invasion and metastasis (Gocheva and Joyce, 2007; Obermajer et al., 2006b) Cathepsin X was found to be upregulated in gastric and prostate

carcinomas, and related to the invasiveness of tumour cells (Kruger et al., 2005; Nagler et al., 2004). However, it has been demonstrated that it does not contribute to ECM degradation or affect ECM-dependent cell migration (Kos et al., 2005; Lechner et al., 2006).

During firm adhesion, the combination of integrin signalling and exposure to immobilised chemokines on the apical surface of endothelial cells induces a marked change in the morphology of T lymphocytes. Migration-associated polarisation is initiated by polarised redistribution of the cell surface receptors and cytoskeletal elements, resulting in the formation of three different morphological and functional compartments: (1) the leading edge with one or several lamellipodia rich in F-actin, chemokine receptors and substrate-adhesion molecules, (2) the mid-cell region and, (3) the uropod which is a distinctive region projecting from the trailing edge (Vincente-Manzanares and Sanchez-Madrid, 2004). The uropod contains multiple cytoskeletal elements, such as microtubule and intermediate-filament networks, F-actin, radixin, moesin, ezrin (Ratner et al., 1997; del Pozo et al., 1997), several adhesion molecules, including CD44, CD43 (Sanchez-Mateos et al., 1995), as well as ICAMs, β1 integrins and LFA-1 (Friedl et al., 1998; del Pozo et al., 1998; Smith et al., 2005). The functions of the uropod are (1) to enhance T lymphocyte migration through the ECM and transendothelial migration (Ratner et al., 1997), (2) to increase transendothelial migration of unpolarised bystander cells through homotypic anchoring and by pulling the cells through the endothelial barrier and underlying ECM and, (3) to mediate ICAM1-dependent interactions of memory T lymphocytes with other cells (del Pozo et al., 1997).

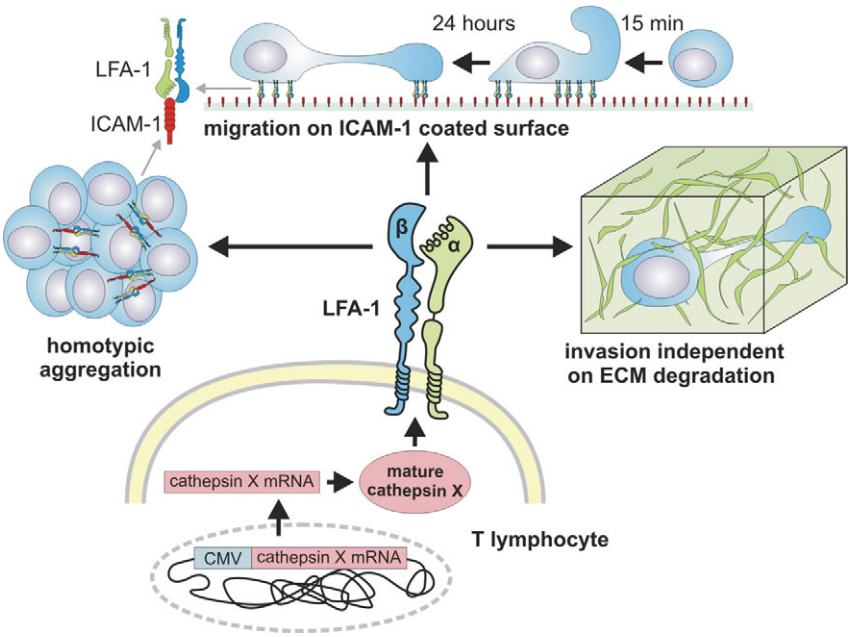

Fig. 9. Potential role of cathepsin X in the aggregation, migration and invasiveness of T lymphocytes. The potential physiological role of cathepsin X was investigated by its overexpression in T lymphocytes. Overexpressed cathepsin X induces morphological changes and promotes migration, invasiveness and homotypic aggregation by modulating the activity of the β2 integrin LFA-1 that is abundantly expressed in T lymphocytes.

We studied the role of cathepsin X in T lymphocyte migration by following migration of the cells across an ICAM1-coated surface by time-lapse microscopy. In contrast to the wild-type Jurkat T lymphocytes, which migrated across the 2D surface in an apparently random manner, the movement of cathepsin-X-overexpressing cells was more dynamic and directed towards each other, resulting in the formation of homotypic aggregates (Movies 1, 2 in supplementary material). A significant portion of the transformed Jurkat T lymphocytes immediately underwent cytoskeletal changes, adopting a polarised phenotype with lamellipodia, the mid-cell and the uropod, whereas the majority of the wild-type cells remained in a spherical non-migratory state. Mature cathepsin-X was found to be colocalised with LFA-1 in polarised cells, especially at the mid-zone and at the uropod, suggesting that cathepsin X has a role in the activation of LFA-1 that is essential for the induction of cytoskeletal changes and migration on ICAM1 (Kelleher et al., 1995). This is further supported by the fact that active LFA-1 was also localised at the mid-zone and at the uropod of cathepsin-X-overexpressing cells. We found that upregulation of cathepsin X increased transmigration of T lymphocytes through uncoated, as well as through ICAM1-coated polycarbonate membranes in a Transwell migration model. Interestingly, the migration on ICAM1 was slower than on uncoated membrane, most probably due to active LFA-1 at the uropod, which caused stronger attachment of the trailing edge. Previous studies revealed that LFA-1, inactive in terms of ICAM1 binding, is located in the uropod, whereas its high-affinity form is located in the mid-cell zone. When LFA-1 is locked in the high-affinity form by activation monoclonal antibody, LFA-1 binding to ICAM1 increases dramatically in the mid-cell zone, as well as in the uropod. The outcome is that the trailing edge becomes firmly attached to the ligand surface (Smith et al., 2005).

Prolonged incubation of cathepsin-X-overexpressing T lymphocytes on ICAM1 resulted in the formation of slim extended uropods. The synergistic effect of cathepsin X and PMA, which promotes LFA-1 clustering (Hogg et al., 2003) caused lymphocyte arrest and attachment of the uropod peak on the ICAM1-coated surface or on proximal cells. Strong colocalisation of cathepsin X and LFA-1 at the attached peaks of extended uropods has been observed (data not shown). We therefore propose that the increased level of cathepsin X promotes constant activation of LFA-1, also at the uropod, presumably by proteolytic modification, which induces a conformational change that increases the affinity of LFA-1. As a lysosomal protease active cathepsin X was colocalised with the lysosomal marker protein LAMP2. However, beside lysosomal localisation active cathepsin X can also be seen in the perimembranous region and in the uropod and might therefore cleave the cytoplasmic tail of the LFA-1 β2-chain. Our recent results show that the 15 amino acid C-terminal peptide of the LFA-1 β2-chain (755KSATTTVMNPKFAES769) can be cleaved sequentially by cathepsin X for four C-terminal amino acids, up to K765, at pH 5.5 and also pH 7.0 (N.O., unpublished). This supports the intracellular action of active cathepsin X.

The migration of transfected T lymphocytes was slower in the presence of 2F12 mAb and the epoxysuccinyl-based cell-permeable inhibitor AMS36, which both neutralise cathepsin X activity. AMS36 showed a stronger effect than 2F12 mAb due to better

internalisation, supporting the cytoplasmic action of cathepsin X on β-chain of LFA-1.

The locomotion of T lymphocytes within the ECM is a highly dynamic and flexible process following the principles of amoeboid movement. Amoeboid motility is characterised by a polarised cell shape allowing high speed, rapid directional oscillations and low-affinity interactions to the substrate, and a lack of proper focal adhesions (Friedl et al., 2001). In our 3D Matrigel model the majority of cathepsin-X-overexpressing cells underwent profound polarisation of the cell shape, with visible uropods and by exhibiting redistribution of cytoskeletal proteins such as α-actinin-1. However, only a few wild-type cells spontaneously developed a polarised morphology (Movies 3, 4 in supplementary material). Morphological changes of cathepsin-X-overexpressing cells where more evident after activation with PMA, suggesting a synergistic effect that was observed also in the 2D ICAM model. In wild-type cells that express low levels of cathepsin X only small morphological changes were visible after treatment with PMA.

Not only migration, but also invasion of cathepsin-X-overexpressing T lymphocytes through the Matrigel barrier, was significantly greater than that of wild-type cells. The role of cathepsin X in proteolytic remodelling of the ECM by cell lines with proven metastatic potential has been excluded in our previous study (Kos et al., 2005). T lymphocytes migrate through ECM by amoeboid movement and do not utilise proteolytic degradation (Friedl et al., 2001). The latter was confirmed in our study since DQ-collagen-IV was not degraded during T-lymphocyte-invasion in 3D Matrigel, whereas the migration of highly invasive MCF-10A neoT cells was accompanied by extensive degradation of the Matrigel. This is further evidence that cathepsin X promotes mobility of T lymphocytes by inducing morphological changes rather than taking part in the proteolytic cleavage of the ECM.

In conclusion, our results show that cathepsin X acts as a promoter of T-lymphocyte migration on 2D and 3D ICAM1 and Matrigel models that mimic the ECM. This is associated with LFA-1 activation, resulting in extensive cytoskeletal rearrangement and cell polarisation (Fig. 9). Cathepsin X might, therefore be an important factor enabling T lymphocytes to function effectively as migratory cells during the immune response.

Materials and Methods

Cell culture

Cells of the Jurkat T lymphocyte line (TIB-152) (ATCC, Manassas, VA) were grown in RPMI 1640 medium (Sigma, St Louis, MO), supplemented with 2 mM glutamine (Sigma), 2 g/l sodium bicarbonate (Riedel de Haën, St Louis, MO), antibiotics and 5% fetal bovine serum (FCS) (HyClone, Logan, UT). MCF-10A neoT human breast epithelial cell line was provided by Bonnie F. Sloane (Wayne State University, Detroit, MI). MCF-10A neoT cells were cultured in DMEM/F12 (1:1) medium (Gibco, Invitrogen, Scotland) supplemented with 1 μg/ml insulin (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 50 ng/ml epidermal growth factor (Sigma-Aldrich), antibiotics and 5% FCS.

Antibodies

Monoclonal antibodies against cathepsin X, 2F12 and 3B10, were prepared from mouse hybridoma cell lines as reported (Kos et al., 2005). 2F12 mAb was used as neutralising antibody to inhibit cathepsin X carboxypeptidase activity in aggregation, migration and invasion assays.

Vector construction

The cDNA for cathepsin X was obtained from RZPD German Resource Center for Genome Research and amplified using the synthetic primers: 5′-GGTAGG-ATCCATTATGGCGAGGCGCGGG-3′ and 5′-CACTCTCGAGCTAAACGAT-GGGGTCCCCAAATG-3′. PCR products were digested with *BamH*I and *Xho*I and inserted into the pcDNA3 vector (Invitrogen), yielding pcDNA3/cathepsin X. The nucleotide sequence of the inserted cathepsin X gene was determined at MWG Biotech DNA sequencing services.

Cell transfection

Jurkat T lymphocytes were seeded in 24-well plates (2×10^5 cells per well), 1 day prior to transfection. The next day, cells were transfected with pcDNA3/cathepsin X construct or with pEGFP-N1 α-actinin 1 (Addgene) using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 15 μg of plasmid DNA re-suspended in 250 μl of RPMI (without FCS or antibiotics) were mixed with 10 μl of lipofectamine in 250 μl of RPMI and incubated for 20 minutes at room temperature. The DNA-lipid mix was added to the cells and incubated at 37°C in 5% CO_2 for 5 hours. The transfection medium was then removed and replaced with complete RPMI 1640 medium. 24 hours after lipofection the cells were diluted and stable lines selected in 400 μg/ml Geneticin (Gibco, Invitrogen, UK). Single-cell clones were isolated from the lipofected Jurkat T lymphocyte population by a limiting-dilution method.

Quantitative ELISA

To prepare cell lysates for the analysis of the cathepsin X protein levels, Jurkat T lymphocytes (5×10^5 cells/sample) were washed with phosphate buffered saline (PBS) pH 7.4 and centrifuged for 5 minutes at 300 ***g***. Pellets was re-suspended in 50 μl of 0.05 M sodium acetate buffer pH 6, 1 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100 and stored at –80°C. Total protein concentration of the samples was determined according to the Bradford method using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories). Cathepsin X ELISA was performed as reported (Kos et al., 2005). Microtiter plates were coated with 5 μg/ml of 2F12 mAb in 0.01 M carbonate/bicarbonate buffer pH 9.6 at 4°C. After blocking (2% BSA-PBS, 150 μl/well), the samples or cathepsin X standards were added (100 μl/well). Following a 2-hour incubation, the wells were washed and filled with 3B10 mAb conjugated to horseradish peroxidase (HRP). After a further 2-hour incubation at 37°C, 200 μg/well of TMB and 0.012% H_2O_2 were added. After 15 minutes, the reaction was stopped by adding 50 μl of 2 M H_2SO_4. The amount of degraded substrate, as a measure of bound immunocomplexed cathepsin X, was determined by measuring the absorbance at 450 nm, and the concentration of cathepsin X was calculated from the calibration curve.

Immunoblotting and active site labelling of cathepsin X in cell lysates

Cell lysates were prepared in lysis buffer (0.05 M sodium acetate, pH 5.5, 1 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100) and protein concentration determined by the Bradford method. Active-site-labelling was preformed as described (Greenbaum et al., 2000; Lennon-Dumenil et al., 2002) using cysteine protease active site directed probe DCG-04. Lysates (100 μg protein per sample) and recombinant active cathepsin X (1 μg protein per sample) were incubated with 0.1 μM DCG-04 for 60 minutes at 37°C. After boiling in reducing (for active site labelling) or non-reducing (for immunoblotting) sample buffer for 10 minutes, samples were analysed by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with PBS/0.5% Tween 20, the membrane was probed with 1 μg/ml streptavidin/horseradish peroxidase (Sigma-Aldrich) or with 10 μg/ml of 2F12 mAb in PBS-0.2% Tween 20 for 60 minutes followed by five washes with PBS/0.2% For immunoblotting, the secondary antibodies were HPR-conjugated goat anti-mouse IgGs (Sigma). The membrane was stained 0.5 mg/ml 3,3′-diaminobenzidine (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.5) and 0.01% (v/v) H_2O_2.

Flow cytometry

Jurkat T lymphocytes (4×10^5 cells) were washed with PBS and incubated with Alexa-Fluor-488 (Beckton Dickinson) conjugated to 2F12 mAb (20 μg/ml) for 30 minutes on ice. Afterwards, the cells were washed with PBS and analysed by flow cytometry for expression of cathepsin X on the cell surface. Alexa-Fluor-488-coupled mouse IgG1 was used as a background control. Flow cytometry was performed using a FACSCalibur system (Becton Dickinson, Inc.).

Quantitative real-time PCR analyses

Total RNA was isolated from Jurkat T lymphocytes using the RNeasy Mini kit (Qiagen, Germany, Hilden) according to the manufacturer's protocol. The RNA was quantified by measuring absorbance at 280 nm on a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE) and the integrity was determined by formaldehyde gel electrophoresis. For cDNA synthesis, 1 μg of total mRNA was reverse transcribed using OmniscriptRT Kit (Qiagen, Germany, Hilden). Quantitative real-time PCR analyses (qPCR) was carried out on an ABI PRISM 7000 apparatus (Applied Biosystems) in a total reaction volume of 25 μl containing 12.5 μl of 1× platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 0.5 μl Rox reference dye, 5 μl cDNA of different concentrations and 0.2 μM of each primer (catx fw, 5′-AAGGAGAAGATGATGGCAGAAA-3′ and catx rev, 5′-TTGCAATTATTC CACAGCTGAT-3′; Invitrogen). The cycling program was as follows: 2 minutes at 50°C, 2 minutes at 95°C, followed by 45 cycles (15 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C). We checked β-actin (ACTB), β2 microglobulin (B2M), hypoxanthine-guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPD), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ) and ubiquitin C (UBC) (the primer sequences were found in the Real Time PCR Primer and Probe Data Base),

housekeeping genes for their stability by using geNorm normalisation and chose the two most stable genes (HPRT and YWHAZ) to be our endogenous controls for qPCR analysis. A melting curve of PCR products (60-95°C) was also performed to ensure the absence of artifacts.

Cell aggregation assay

Stably transfected Jurkat T lymphocytes and wild-type Jurkat T lymphocytes (6×10^4 cells/500 μl) were added to a 8-well LabTek chambered coverglass system (Nalge Nunc International) and incubated at 37°C at 5% CO_2 for 24 hours. The cathepsin X inhibitor AMS36, or 2F12 mAb or EDTA were added to a final concentration of 3 μM (AMS36), 1 μM (2F12 mAb) and 0.25 mM (EDTA), respectively. Transmission microscopy was performed with an Olympus IX 81 motorised inverted microscope and CellR software.

Migration on ICAM1

Stably transfected Jurkat T lymphocytes and wild-type Jurkat T lymphocytes were activated by 50 nM phorbol 12-myristate 13-acetate (PMA) for 48 hours at 37°C. The cells observed by video microscopy were not activated. The wells of an 8-well LabTek chambered coverglass system (Nalge Nunc International) were coated with 10 μg/ml chimeric ICAM1-Fc fusion protein overnight at 4°C Slides were blocked with 3% BSA in PBS for 30 minutes at room temperature and washed with PBS. 400 μl of cell suspension (6×10^4 cells/ml) were added and incubated at 37°C with 5% CO_2 for 24 hours. For time-lapse imaging, 500 μl of cell suspension (8×10^4 cells/ml) was added and allowed to settle for 10 minutes at 37°C, 5% CO_2. Cells were tracked at 30-second intervals for 2 hours. Images were taken using an Olympus IX 81 motorised inverted microscope and CellR software.

Transwell migration assay

The upper side of 8-μm-pore polycarbonate filters of a 24-well Transwell plate (Corning Costar) was either left uncoated or coated overnight with 10 μg/ml chimeric ICAM1-Fc fusion protein (R&D Systems, Minneapolis) at 4°C, and then blocked with 1.5 % BSA in PBS for 30 minutes. The lower side of the filters was coated with 20 μl of fibronectin (50 μg/ml) and incubated for 1 hour. 4×10^5 cathepsin-X-overexpressing Jurkat T lymphocytes or untransfected Jurkat T lymphocytes were suspended in 100 μl of medium and added to the upper compartments. The lower compartments were filled with 600 μl of medium. Cathepsin X epoxysuccinyl-based inhibitor AMS36 or 2F12 mAb was added to a final concentration of 2 μM (AMS36) or 1 μM (2F12 mAb) to the upper and lower compartments. Transwells were incubated for 24 hours at 37°C and 5% CO_2. All assays were performed in a triplicate. Migration was determined by cell counting and calculated as: cell migration (in %) = $N_{lower}/(N_{lower} + N_{upper}) \times 100$, where N represents the average cell number in each compartment.

Migration on Matrigel

The wells of a 8-well LabTek chambered coverglass system (Nalge Nunc International) were coated with 50 μl of 100% Matrigel (BD Biosciences) and allowed to solidify for 20 minutes at 37°C. Jurkat T lymphocytes were transfected with pEGFP-N1 alpha-actinin 1 and seeded the next day onto Matrigel as a single-cell suspension (5×10^4 cell/ml) in complete RPMI 1640 medium containing 2% Matrigel. Cells were incubated for 48 hours at 37°C and 5% CO_2. For time-lapse imaging 500 μl of cell suspension (7×10^4 cells/ml) were added and allowed to settle for 24 hours at 37°C with 5% CO_2. Cells were tracked at 1.5-minute intervals for 16 hours. Images were taken using Olympus IX 81 motorised inverted microscope and CellR software.

Transwell invasion assay

The lower side of 8-μm-pore polycarbonate filters of a 24-well Transwell plate (Corning Costar) was coated with 20 μl of fibronectin (50 μg/ml) and incubated for 1 hour. The upper side of the filters was coated with 50 μl of 3.4 mg/ml Matrigel (BD Biosciences) and incubated at room temperature for 1 hour. 4×10^5 of cathepsin-X-overexpressing Jurkat T lymphocytes or untransfected Jurkat T lymphocytes were suspended in 100 μl of medium and added to the upper compartments. The lower compartments were filled with 600 μl of medium. The athepsin X inhibitor AMS36, or 2F12 mAb were added to a final concentration of 2 μM (AMS36) or 1 μM (2F12 mAb) to the upper and lower compartments. Transwells were incubated for 24 hours at 37°C and 5% CO_2. All assays were performed in a triplicate. Invasion was determined by cell counting and calculated as: cell invasion (in %)=$N_{lower}/(N_{lower} + N_{upper}) \times 100$, where N represents the average cell number in each compartment.

Degradation of DQ-collagen-IV by living cells

The wells of LabTek chambered coverglass system (Nalge Nunc International) were coated with 25 μg/ml of the quenched fluorescent substrate DQ-collagen-IV (Molecular Probes, Invitrogen) suspended in 50 μl of 100% Matrigel (BD Biosciences), for 20 minutes at 37°C. 400 μl of Jurkat T lymphocytes or MCF-10A neoT cells were seeded onto Matrigel as a single-cell suspension (3×10^4 cell/ml) in assay-medium containing 2% Matrigel. Cells were incubated for 48 hours at 37°C with 5% CO_2 and monitored for fluorescent degradation products of DQ-collagen-IV (Olympus IX 81 motorised inverted microscope and CellR software).

Immunofluorescence microscopy

Jurkat T lymphocytes were seeded on ICAM1-Fc pre-coated slides and allowed to adhere for 15 minutes. Afterwards, the slides were cytospined for 6 minutes at 1300 rpm. Before labelling, the cells were fixed with methanol (–20°C) or 4% solution of paraformaldehide in PBS for 10 minutes and permeabilised by 0.01% Triton X-100 in PBS for 10 minutes. Non-specific staining was blocked with 3% BSA in PBS pH 7.4, for 1 hour. Jurkat T lymphocytes grown on Matrigel (for 48 hours) were fixed with 2% solution of formaldehide in PBS for 20 minutes at room temperature and permeabilised by 0.25% Triton X-100 in PBS pH 7.4, for 10 minutes at 4°C. Cells were rinsed three times with PBS/glycine (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 100 mM glycine; 15 minutes per wash at room temperature). Non-specific staining was blocked with 3% BSA in PBS, 0.01% Triton X-100, 0.05% Tween-20) for 1.5 hours. Cathepsin X was labelled with Alexa-Fluor-488 (Molecular Probes, Carlsbad, CA) conjugated to mouse 2F12 mAb (10 μg/ml), which recognises the mature form, for 2 hours at room temperature overnight at 4°C, 200 μl/well for Matrigel cultures. For integrin labelling, the primary antibodies were goat anti-human integrin LFA-1 N-18 (5 μg/ml) from Santa Cruz Biotechnology (Santa Cruz, CA). For labelling of active LFA-1, the primary antibody was mAb 24 provided by Nancy Hogg (Macrophage Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK). The cells were washed with PBS (or 3% BSA in PBS for Matrigel cultures) and treated with Alexa-Fluor-633-labelled donkey anti-goat or Alexa-Fluor-488-labelled rabbit anti-mouse secondary antibody (Molecular Probes) for 2 hours. For lysosomal colocalisation LAMP-2 was labelled with the primary mouse monoclonal antibody H4B4 anti-human-LAMP-2 (BD Pharmingen, Germany) and Alexa-Fluor-546-labelled goat anti-mouse secondary antibody. After washing three times with PBS (15 minutes per wash) cathepsin X was labelled with Alexa-Fluor-488-labelled mouse 2F12 mAb. After washing with PBS (or 3% BSA in PBS for Matrigel cultures), ProLong Antifade kit (Molecular Probes, Carlsbad, CA) was mounted on dried slides and allow to dry overnight at room temperature. Fluorescence microscopy was performed using a Carl Zeiss LSM 510 confocal microscope. Alexa-Fluor-488, Alexa-Fluor-546 and Alexa-Fluor-633 were excited with an Arg (488 nm) or He-Ne (546 nm and 633 nm) laser and emission was filtered using a narrow band LB 505-530 nm, LP 560 nm and LP 650 nm filter, respectively. Images were analysed using Carl Zeiss LSM image software 3.0.

Statistical analysis

SPSS PC software (Release 13.0) was used for statistical analysis. The difference between the groups was evaluated using the non-parametric Mann-Whitney test. P values of <0.05 were considered to be statistically significant.

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References

Beauvais, D. M., Burbach, B. J. and Rapraeger, A. C. (2004). The syndecan-1 ectodomain regulates alphavbeta3 integrin activity in human mammary carcinoma cells. *J. Cell Biol.* **167**, 171-181.

Buhling, F., Peitz, U., Kruger, S., Kuster, D., Vieth, M., Gebert, I., Roessner, A., Weber, E., Malfertheiner, P. and Wex, T. (2004). Cathepsins K, L, B, X and W are differentially expressed in normal and chronically inflamed gastric mucosa. *Biol. Chem.* **385**, 439-445.

Campanero, M. R., del Pozo, M. A., Arroyo, A. G., Sanchez-Mateos, P., Hernandez-Caselles, T., Craig, A., Pulido, R. and Sanchez-Madrid, F. (1993). ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway. *J. Cell Biol.* **123**, 1007-1016.

Coates, T. D., Watts, R. G., Hartman, R. and Howard, T. H. (1992). Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. *J. Cell Biol.* **117**, 765-774.

de Fougerolles, A. R. and Springer, T. A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med.* **175**, 185-190.

del Pozo, M. A., Cabanas, C., Montoya, M. C., Ager, A., Sanchez-Mateos, P. and Sanchez-Madrid, F. (1997). ICAMs redistributed by chemokines to cellular uropods as a mechanism for recruitment of T lymphocytes. *J. Cell Biol.* **137**, 493-508.

del Pozo, M. A., Nieto, M., Serrador, J. M., Sancho, D., Vicente-Manzanares, M., Martinez, C. and Sanchez-Madrid, F. (1998). The two poles of the lymphocyte: specialized cell compartments for migration and recruitment. *Cell Adhes. Commun.* **6**, 125-133.

Dransfield, I., Cabanas, C., Craig, A. and Hogg, N. (1992). Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J. Cell Biol.* **116**, 219-226.

Dustin, M. L., Carpen, O. and Springer, T. A. (1992). Regulation of locomotion and cell-cell contact area by the LFA-1 and ICAM-1 adhesion receptors. *J. Immunol.* **148**, 2654-2663.

Friedl, P. and Brocker, E. B. (2000). T cell migration in three-dimensional extracellular matrix: guidance by polarity and sensations. *Dev. Immunol.* **7**, 249-266.

Friedl, P., Entschladen, F., Conrad, C., Niggemann, B. and Zanker, K. S. (1998). CD4+ T lymphocytes migrating in three-dimensional collagen lattices lack focal adhesions and utilize beta1 integrin-independent strategies for polarization, interaction with collagen fibers and locomotion. *Eur. J. Immunol.* **28**, 2331-2343.

Friedl, P., Borgmann, S. and Brocker, E. B. (2001). Amoeboid leukocyte crawling through extracellular matrix: lessons from the Dictyostelium paradigm of cell movement. *J. Leukoc. Biol.* **70**, 491-509.

Gocheva, V. and Joyce, J. A. (2007). Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle* **6**, 60-64.

Greenbaum, D., Medzihradszky, K. F., Burlingame, A. and Bogyo, M. (2000). Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol*. **7**, 569-581.

Gunčar, G., Klemenčič, I., Turk, B., Turk, V., Karaoglanovic-Carmona, A., Juliano, L. and Turk, D. (2000). Crystal structure of cathepsin X: a flip-flop of the ring of His23 allows carboxy-monopeptidase and carboxy-dipeptidase activity of the protease. *Structure* **8**, 305-313.

Hogg, N., Laschinger, M., Giles. and K., McDowall, A. (2003). T-cell integrins: more than just sticking points. *J. Cell Sci.* **116**, 4695-4705.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Kelleher, D., Murphy, A., Feighery, C. and Casey, E. B. (1995). Leukocyte function-associated antigen 1 (LFA-1) and CD44 are signalling molecules for cytoskeleton-dependent morphological changes in activated T cells. J. *Leukoc. Biol.* **58**, 539-546.

Klemenčič, I., Carmona, A. K., Cezari, M. H., Juliano, M. A., Juliano, L., Guncar, G., Turk, D., Krizaj, I., Turk, V. and Turk, B. (2000). Biochemical characterization of human cathepsin X revealed that the enzyme is an exopeptidase, acting as carboxymonopeptidase or carboxydipeptidase. *Eur. J. Biochem.* **267**, 5404-5412.

Kokkonen, N., Rivinoja, A., Kauppila, A., Suokas, M., Kellokumpu, I. and Kellokumpu, S. (2004). Defective acidification of intracellular organelles results in aberrant secretion of cathepsin D in cancer cells. *J Biol. Chem.* **279**, 39982-39988.

Kos, J., Sekirnik, A., Premzl, A., Zavasnik-Bergant, V., Langerholc, T., Turk, B., Werle, B., Golouh, R., Repnik, U., Jeras, M. et al. (2005). Carboxypeptidases cathepsins X and B display distinct protein profile in human cells and tissues. *Exp. Cell Res.* **306**, 103-113.

Krueger, S., Kalinski, T., Hundertmark, T., Wex, T., Kuster, D., Peitz, U., Ebert, M., Nagler, D. K., Kellner, U., Malfertheiner et al. (2005). Up-regulation of cathepsin X in *Helicobacter pylori* gastritis and gastric cancer. *J. Pathol.* **207**, 32-42.

Labadia, M. E., Jeanfavre, D. D., Caviness, G. O. and Morelock, M. M. (1998). Molecular regulation of the interaction between leukocyte function-associated antigen-1 and soluble ICAM-1 by divalent metal cations. *J. Immunol*. **161**, 836-842.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Lechner, A. M., Assfalg-Machleidt, I., Zahler, S., Stoeckelhuber, M., Machleidt, W., Jochum, M. and Nagler, D. K. (2006). RGD-dependent binding of procathepsin X to integrin alphavbeta3 mediates cell-adhesive properties. *J. Biol. Chem.* **281**, 39588-39597.

Lennon-Dumenil, A. M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L. and Lagaudriere-Gesbert, C. (2002). Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* **196**, 529-540.

Liliental, J. and Chang, D. D. (1998). Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. *J. Biol. Chem.* **273**, 2379-2383.

Longhurst, C. M. and Jennings, L. K. (1998). Integrin-mediated signal transduction. *Cell Mol. Life Sci.* **54**, 514-526.

Marlin, S. D. and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* **51**, 813-819.

Menard, R., Therrien, C., Lachance, P., Sulea, T., Qo, H., Alvarez-Hernandez, A. D. and Roush, W. R. (2001). Cathepsins X and B display distinct activity profiles that can be exploited for inhibitor design. *Biol. Chem.* **382**, 839-845.

Nagler, D. K. and Menard, R. (1998). Human cathepsin X: a novel cysteine protease of the papain family with a very short proregion and unique insertions. *FEBS Lett.* **434**, 135-139.

Nagler, D. K., Zhang, R., Tam, W., Sulea, T., Purisima, E. O. and Menard, R. (1999). Human cathepsin X: A cysteine protease with unique carboxypeptidase activity. *Biochemistry* **38**, 12648-12654.

Nagler, D. K., Kruger, S., Kellner, A., Ziomek, E., Menard, R., Buhtz, P., Krams, M., Roessner, A. and Kellner, U. (2004). Up-regulation of cathepsin X in prostate cancer and prostatic intraepithelial neoplasia. *Prostate* **60**, 109-119.

Nascimento, F. D., Rizzi, C. C., Nantes, I. L., Stefe, I., Turk, B., Carmona, A. K., Nader, H. B., Juliano, L. and Tersariol, I. L. (2005). Cathepsin X binds to cell surface heparan sulfate proteoglycans. *Arch. Biochem. Biophys.* **436**, 323-332.

Obermajer, N., Doljak, B. and Kos, J. (2006). Cysteine cathepsins: regulators of antitumour immune response. *Expert Opin. Biol. Ther.* **6**, 1295-1309.

Obermajer, N., Premzl, A., Zavasnik-Bergant, T., Turk, B. and Kos, J. (2006). Carboxypeptidase cathepsin X mediates beta2-integrin-dependent adhesion of differentiated U-937 cells. *Exp. Cell Res.* **312**, 2515-2527.

Obermajer, N., Repnik, U., Jevnikar, Z., Kreft, M. and Kos, J. (2008). Immunosupressive activity of cysteine protease cathepsin X via activation of beta-2 integrins. *Immunology* **124,** 76-88.

Paulsson, M. (1992). Basement membrane proteins: structure, assembly, and cellular interactions. *Crit. Rev. Biochem. Mol. Biol.* **27**, 93-127.

Petruzzelli, L., Maduzia, L. and Springer, T. A. (1998). Differential requirements for LFA-1 binding to ICAM-1 and LFA-1-mediated cell aggregation. *J. Immunol.* **160**, 4208-4216.

Porter, J. C., Bracke, M., Smith, A., Davies, D. and Hogg, N. (2002). Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. *J. Immunol.* **168**, 6330-6335.

Ratner, S., Patrick, P. and Bora, G. (1992). Lymphocyte development of adherence and motility in extracellular matrix during IL-2 stimulation. *J. Immunol.* **149**, 681-688.

Ratner, S., Sherrod, W. S. and Lichlyter, D. (1997). Microtubule retraction into the uropod and its role in T cell polarization and motility. *J. Immunol.* **159**, 1063-1067.

Sadaghiani, A. M., Verhelst, S. H., Gocheva, V., Hill, K., Majerova, E., Stinson. S., Joyce. J. A. and Bogyo, M. (2007). Design, synthesis, and evaluation of in vivo potency and selectivity of epoxysuccinyl-based inhibitors of papain-family cysteine proteases. *Chem Biol.* **14**, 499-511.

Sanchez-Madrid, F. and del Pozo, M. A. (1999). Leukocyte polarization in cell migration and immune interactions. *EMBO J.* **18**, 501-511.

Sanchez-Mateos, P., Campanero, M. R., del Pozo, M. A. and Sanchez-Madrid, F. (1995). Regulatory role of CD43 leukosialin on integrin-mediated T-cell adhesion to endothelial and extracellular matrix ligands and its polar redistribution to a cellular uropod. *Blood* **86**, 2228- 22239.

Santamaria, I., Velasco, G., Pendas, A. M., Fueyo, A. and Lopez-Otin, C. (1998). Cathepsin Z, a novel human cysteine proteinase with a short propeptide domain and a unique chromosomal location. *J. Biol. Chem.* **273**, 16816-16823.

Sivaraman, J., Nagler, D. K., Zhang, R., Menard, R. and Cygler, M. (2000). Crystal structure of human procathepsin X: a cysteine protease with the proregion covalently linked to the active site cysteine. *J. Mol. Biol.* **295**, 939-951.

Smith, A., Carrasco, Y. R., Stanley, P., Kieffer, N., Batista, F. D. and Hogg, N. (2005). A talin-dependent LFA-1 focal zone is formed by rapidly migrating T lymphocytes. *J. Cell Biol.* **170**, 141-151.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314.

Stanley, P. and Hogg, N. (1998). The I domain of integrin LFA-1 interacts with ICAM-1 domain 1 at residue Glu-34 but not Gln-73. *J. Biol. Chem.* **273**, 3358-3336.

Staunton, D. E., Dustin, M. L. and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature* **339**, 61-64.

Staunton, D. E., Dustin, M. L., Erickson, H. P. and Springer, T. A. (1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* **61**, 243-254.

Stewart, M. P., Cabanas, C. and Hogg, N. (1996). T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. *J. Immunol.* **156**, 1810-1817.

Stewart, M. P., McDowall, A. and Hogg, N. (1998). LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. *J. Cell Biol.* **140**, 699-707.

van Andrian, U. H. and Mackay, C. R. (2000). T-cell function and migration. Two sides of the same coin. *N. Engl. J. Med.* **343**, 1020-1034.

van der Flier, A. and Sonnenberg, A. (2001). Function and interactions of integrins. *Cell Tissue Res.* **305**, 285-298.

van Kooyk, Y., Weder, P., Heije, K. and Figdor, C. G. (1994). Extracellular Ca2+ modulates leukocyte function-associated antigen-1 cell surface distribution on T lymphocytes and consequently affects cell adhesion. *J. Cell Biol.* **124**, 1061-1070.

Varga, G., Balkow, S., Wild, M. K., Stadtbaeumer, A., Krummen, M., Rothoeft, T., Higuchi, T., Beissert, S., Wethmar, K., Scharffetter-Kochanek, K. et al. (2007). Active MAC-1 (CD11b/CD18) on DCs inhibits full T-cell activation. *Blood* **109**, 661-669.

Verhelst, S. H. and Bogyo, M. (2005). Solid-phase synthesis of double-headed epoxysuccinyl activity-based probes for selective targeting of papain family cysteine proteases. *Chembiochem.* **6**, 824-827.

Vicente-Manzanares, M. and Sanchez-Madrid, F. (2004). Role of the cytoskeleton during leukocyte responses. *Nat. Rev. Immunol.* **4**, 110-122.

Wendt, W., Zhu, X. R., Lubbert, H. and Stichel, C. C. (2007). Differential expression of cathepsin X in aging and pathological central nervous system of mice. *Exp. Neurol.* **204**, 525-540.

Wolf, K., Muller, R., Borgmann, S., Brocker, E. B. and Friedl, P. (2003). Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. *Blood* **102**, 3262-3269.