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

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Accepted 19 May 2008
Journal of Cell Science 121, 2652-2661 Published by The Company of Biologists 2008
doi:10.1242/jcs.023721

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.

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 (Guncar 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’ (Guncar et al., 2000). Cathepsin X exhibits mono- di-peptidase activity at its C-terminus (Guncar 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 cellular 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 αβ3 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 (Labadía 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
Under time-lapse microscopy cathepsin-X-overexpressing cells started immediately to move towards each other, resulting in the 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.

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 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%.

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.
Cathepsin X in T lymphocyte migration

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 mainly 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 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-
X-overexpressing cells. The cytoskeletal rearrangements were followed by the GFP-linked cytoskeletal protein \( \alpha \)-actinin-1 (Fig. 3C). In wild-type cells, GFP–\( \alpha \)-actinin-1 was distributed throughout the cells, being more concentrated in the forming lamellipodium. In cathepsin-X-overexpressing cells the accumulation of GFP–\( \alpha \)-actinin-1 at the lamellipodium is more evident. Moreover, GFP–\( \alpha \)-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 \( \beta \)2 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
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 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 Mn2+ or Mg2+ (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 increases 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 (Krugler 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).

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 (755KSATTTVMPNPKFAES769) 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 epoxyavulin-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 ICAM1 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% FCS. 50 ng/ml hydrocortisone (Sigma-Aldrich), 50 ng/ml epidermal growth factor (Sigma-Aldrich), 0.5 μg/ml insulin (Sigma-Aldrich), 0.5 μg/ml streptavidin/horseradish peroxidase (Sigma-Aldrich) or with 100 ng/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 × 10^6 cells/sample) were washed with phosphate buffered saline (PBS) pH 7.4 and centrifuged for 5 minutes at 300 g. Pellets were 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% H2O2 were added. After 15 minutes, the reaction was stopped by adding 50 μl of 2 M H2SO4. 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 performed as described (Greenbaum et al., 2000; Lennon-Duménil et al., 2002) using cysteine protease active site directed probe DCG-04. Lysates (100 μg protein per sample) and recombiant 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 membranes were developped for 1 hour using ECL (Amersham). The membrane was stained 0.5 mg/ml 3,3′-diaminobenzidine (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.5) and 0.1% (v/v) H2O2.

Flow cytometry
Jurkat T lymphocytes (4 × 10^5 cells) were washed with PBS and incubated with Alexa-Flour-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-Flour-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′-AAGGAGAGATGATGCGGAGCAAA-3′ and catx rev, 5′-TGGCAATATTCCACACGGCGAT-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), B2 microglobulin (B2M), hypoxanthine-guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPD), tyrosinase 3-monoxygenase/tryptophan 5-monoxygenase activation protein (YWHAZ) and ubiquitin C (UBC) (the primer sequences were found in the Real Time PCR Primer and Probe Database, GenBank: DNA sequencing services.
housekeeping genes for their stability by using g35Norm normalisation and chose the two most stable genes (HPRF 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 × 10⁴ cells/500 μl) were added to a 8-well LabTek chambered coverglass system (Nalge Nunc International) and incubated at 37°C at 5% CO₂ for 24 hours. The cathepsin X inhibitor AMS36, or 2F12 mAb was added to a final concentration of 2 μM overexpressing Jurkat T lymphocytes or untransfected Jurkat T lymphocytes were suspended in 100 μl of medium and incubated at 37°C for 24 hours at 37°C and 5% CO₂. Cells were tracked at 1.5-minute intervals for 16 hours. Images were taken using 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 × 10⁴ cells/ml) were added and incubated at 37°C with 5% CO₂ for 24 hours. For time-lapse imaging, 500 μl of cell suspension (8 × 10⁴ cells/ml) was added and allowed to settle for 10 minutes at 37°C, 5% CO₂. 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 μg of fibronectin (500 μg/ml) and incubated for 1 hour. 4 × 10⁵ 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 oxopsycosinyl-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% CO₂. All assays were performed in a triplicate. Migration was determined by cell counting and calculated as: cell migration (in %) = Nlower/(Nlower + Nupper) × 100, where N represents the average cell number in each compartment.

Migration on Matrigel
The wells of an 8-well LabTek chambered coverglass system (Nalge Nunc International) were coated with 50 μl of Matrigel (BD Biosciences) and incubated at room temperature for 24 hours. For time-lapse imaging, 500 μl of cell suspension (5 × 10⁵ cells/ml) in complete RPMI 1640 medium containing 2% Matrigel. Cells were incubated for 48 hours at 37°C and 5% CO₂. Cells were incubated for 48 hours at 37°C and 5% CO₂. For time-lapse imaging 500 μl of cell suspension (7 × 10⁴ cells/ml) in complete RPMI 1640 medium containing 2% Matrigel. Cells were incubated for 48 hours at 37°C and 5% CO₂. The 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 oxopsycosinyl-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% CO₂. All assays were performed in a triplicate. Migration was determined by cell counting and calculated as: cell migration (in %) = Nlower/(Nlower + Nupper) × 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 × 10⁵ cell/ml) in assay-medium containing 2% Matrigel. Cells were incubated for 48 hours at 37°C with 5% CO₂ 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 cytofixed for 6 minutes at 1300 rpm. Before labelling, the cells were fixed with methanol (−20°C) or 4% solution of parafomaldehyde 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 for 1 hour. Transwell invasion assay
Transwells were incubated for 24 hours at 37°C and 5% CO₂. All assays were performed in a triplicate. Invasion was determined by cell counting and calculated as: invasion (in %) = Nlower/(Nlower + Nupper) × 100, where N represents the average cell number in each compartment.

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.

We thank Roger Pain for the critical reading of the manuscript and Nancy Hogg for the generous gift of mAb 24. This work was supported by the Research Agency of the Republic of Slovenia (J.K.), the 6th EU Framework IP project CancerDegradome (I.K.) and NIH grant U54RR020843 (M.B.).

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Cathespin X in T lymphocyte migration

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