Effector granules in human T lymphocytes: the luminal proteome of secretory lysosomes from human T cells

Hendrik Schmidt1, Christoph Gelhaus2, Melanie Nebendahl1, Marcus Lettau1, Ralph Lucius3, Matthias Leippe2, Dietrich Kabelitz1, Ottmar Janssen1*

Abstract

Background: Cytotoxic cells of the immune system have evolved a lysosomal compartment to store and mobilize effector molecules. In T lymphocytes and NK cells, the death factor FasL is one of the characteristic marker proteins of these so-called secretory lysosomes, which combine properties of conventional lysosomes and exocytotic vesicles. Although these vesicles are crucial for immune effector function, their protein content in T cells has so far not been investigated in detail.

Results: In the present study, intact membranous vesicles were enriched from homogenates of polyclonally activated T cells and initially characterized by Western blotting and electron microscopic inspection. The vesicular fraction that contained the marker proteins of secretory lysosomes was subsequently analyzed by 2D electrophoresis and mass spectrometry. The proteome analysis and data evaluation revealed that 70% of the 397 annotated proteins had been associated with different lysosome-related organelles in previous proteome studies.

Conclusion: We provide the first comprehensive proteome map of T cell-derived secretory lysosomes with only minor contaminations by cytosolic, nuclear or other proteins. This information will be useful to more precisely address the activation-dependent maturation and the specific distribution of effector organelles and proteins in individual T or NK cell populations in future studies.

Background

Cytotoxic T lymphocytes (CTL) and Natural Killer (NK) cells are the main cytotoxic effector cells of the immune system. In order to effectively eliminate virus-infected and tumorigenic cells, they rapidly mobilize effector molecules including granzymes, perforin, granulysin and the death factor FasL (CD178) that are presumably stored in preformed organelles termed secretory lysosomes (SL) [1]. Secretory lysosomes combine degradative properties of conventional lysosomes with characteristics of exocytotic vesicles. At the level of morphology, conventional and secretory lysosomes are hardly distinguishable and both appear to represent endpoints of an endocytotic pathway and are formed by fusion and fission of endosomes and lysosomes [2].

Similar to conventional lysosomes, large membrane areas are covered by lysosome-associated membrane-proteins (LAMPs) including LAMP-1 (CD107a), LAMP-2 (CD107b) and LAMP-3 (CD63) [3-5]. However, secretory effector lysosomes are characterized by a specific set of membrane and luminal marker proteins [6,7]. The current consensus is that SL of CTLs and NK cells carry the aforementioned effector proteins either in the lysosomal lumen (granzymes, perforin and granulysin) or as characteristic transmembrane compounds (FasL) [8-10].

Recently, we provided a protocol that allows a substantial enrichment of intact SL from in vitro expanded lymphocyte populations [11]. Employing this procedure for subcellular fractionation of a crude organelle preparation, we obtained a fraction of intact vesicles that is significantly enriched in SL marker proteins. We were thus able to report the first comprehensive analysis of the luminal proteome of secretory lysosomes from NK cells [12]. At that time, 234 different proteins were
identified by mass spectrometry, 77% of which had been associated with SL or other lysosomal compartments before. Applying 2D difference gel electrophoresis, we also described a cell line-specific distribution of functionally relevant proteins in SL from human NK cell lines and primary NK cells [12].

Based on this study, it appears likely that different T cell populations utilize the SL organelles to store and mobilize lineage-specific cargo proteins. However, the proteome of secretory lysosomes in T cells has not been deciphered. To provide the first proteome map for T cell-derived SL, we enriched organelles from activated T lymphoblasts. Organelle extracts were subjected to SDS-PAGE and Western blotting to identify the FasL-containing SL fraction. This fraction was analyzed by electron microscopy to demonstrate the enrichment of a homogeneous population of intact vesicles. In order to define the luminal proteome of the respective SL compartment, the organelles were lysed and proteins were separated by 2D gel electrophoresis. Mass spectrometry was applied to identify individual spots. We annotated 397 proteins, 70% of which had been associated with lysosome-related organelles before. With the present report, we thus provide the first comprehensive description of the content of FasL-carrying effector vesicles isolated from activated human T lymphocytes.

Results and Discussion
In our preceding analysis of the SL compartment of NK cell lines and primary NK cells, we annotated 234 individual proteins and demonstrated a cell line-specific distribution of several functionally relevant molecules including cytotoxic effector proteins, lysosomal proteases and MHC molecules [12]. As a basis to address unsolved issues regarding the maturation, function and cell type-specific composition of the cytotoxic effector compartment in T cell populations, we now analyzed the proteome of enriched secretory lysosomes from in vitro activated human T cell blasts.

FasL-associated secretory lysosomes in activated lymphocytes
We and others have shown that in CTLs, preformed FasL accumulates in the limiting membrane of secretory lysosomes with late endosome or multi-vesicular-body structure and there co-localizes with characteristic lysosomal marker proteins including CD63 or lysosomal hydrolases and cytoskeletal adapter proteins [7-9,13-15]. Confocal laser-scanning microscopy (CLSM) was applied to confirm that FasL also might serve as a marker for secretory lysosomes in in vitro expanded PHA-stimulated T lymphocytes used in the present study. As depicted in Figure 1, we detected an apparent co-localization of CD63 with FasL, granzyme A and the lysosomal protease cathepsin B. It should be mentioned that a common or distinct localization of LAMP-3 (CD63) and FasL is still controversially discussed. Several reports suggest a co-localization of FasL with granule proteins, such as cathepsin D, CD63, granzyme B, perforin and LAMP-1 in a single granular entity [8,9] whereas other studies indicate that CD63 and FasL are located in distinct subcellular compartments [16].

Our protocol for the enrichment of secretory lysosomes yielded six separate fractions that were subjected to further analysis by Western blotting or 2D gel electrophoresis. To demonstrate an effective enrichment of the SL fraction, we first separated the proteins of individual fractions by SDS-PAGE and stained for characteristic organelle marker proteins after Western blotting. As shown in Figure 2, indicated by the high abundance of FasL, CD63 and cathepsin D, SL were enriched in fraction 2. Although LAMP-1 was also enriched in this fraction, the presence of this lysosomal membrane protein in other fractions might indicate the complex composition of the lysosomal compartment in general and that other lysosome-related vesicles might exist with distinct

Figure 1 In T cell blasts, FasL associates with lysosomal vesicles. PHA blasts (d14) were fixed and stained for FasL with NOK1 and Alexa Fluor488-conjugated donkey anti-mouse IgG or for granzyme A with GrA-11 FITC-conjugated mAbs or for cathepsin B with polyclonal goat anti-cathepsin B (N-19) antibodies and Alexa Fluor488-conjugated donkey anti-goat IgG. After extensive washing, all samples were stained for CD63 with Alexa Fluor555-conjugated mAb MEM-259. Nuclei were visualized by DAPI (bar: 10 μm).
biophysical properties that separate at different media densities. As further indicators for the effective organelle enrichment and separation, we used cytochrome oxidase subunit IV (CoxIV) as a marker for mitochondria (see enriched organelles and fraction 5 in Figure 2) and pan-cadherin as a marker for the plasma membrane (only present in whole cell lysates). Of note, all proteins that were enriched in separate fractions were of course also present in the enriched organelle (EO) fraction placed on the gradient. However, due to the relatively low abundance of individual proteins in the EO fraction, Western blot detection at the displayed exposure time did only reveal very faint bands. This is in agreement with our previous report [11] in which we showed a massive enrichment of FasL in fraction 2 while in the starting EO material from different T cell populations, FasL was almost not detectable at the same exposure time.

Regarding the “purity” of the obtained fraction, it should be stressed that most if not all enrichment protocols published so far do not allow a “purification” rather than an “enrichment” of a given organelle population. This is presumably based on the fact that lysosome formation and protein loading is a highly dynamic process that implies fusion and fission of several membranous compartments and a complex protein sorting and transport machinery. For the initial characterization of enriched SL [11], we already pointed to potential “contaminations” in fraction 2, using antibodies against EEA1, a putative marker for endosomes, or Bip/Grp78, a marker for ER, respectively. Interestingly, during these analyses, golgin, a marker for the golgi apparatus/cisternae was only detected in fractions 3-6, but not in fractions 1 and 2 [11]. For the present study, we thus restricted ourselves to routinely check for the marker proteins depicted in Figure 2.

The enriched SL fraction consists of homogeneous intact vesicles
In addition to the biochemical analysis of the individual fractions, we visualized the obtained lysosomal fraction 2 by electron microscopy in comparison to the putative mitochondrial fraction 5. Figure 3 provides characteristic overview pictures of the two fractions. In both cases, the organelles within one fraction display a high degree of homogeneity with respect to their morphology (Figure 3A,C). At higher magnification, the characteristics of the organelles in fraction 2 become apparent. These membranous vesicles are round-shaped with a maximum size of about 700 nm and display a characteristic electron density. In contrast, organelles of fraction 5 are characterized by irregular internal membranous structures (Figure 3B,D) as expected for mitochondria.

The luminal proteome of enriched SL as analyzed by 2D-PAGE and mass spectrometry
In order to obtain a comprehensive list of putative luminal proteins of secretory lysosomes, enriched fraction 2 vesicles of PHA-stimulated T lymphoblasts were subjected to 2D-PAGE. More than 1600 spots from 6 replicate gels were subsequently subjected to proteolytic

Figure 3 Electron micrographs of fractions 2 and 5. Enriched organelles from PHA blasts corresponding to fractions 2 (A, B) and 5 (C, D) were examined under an electron microscope. Overview pictures are given in A and C, magnified areas are shown in B and D. (scale as indicated).
cleavage and lead to the mass spectrometric identification of 1335 spots. Due to repetitive identifications at respective spot locations in different gels, the actual number of identified individual spots decreased to 742. The resulting proteome map is shown as an overview in Figure 4. Additional information on identified proteins and images of individual quadrants to match proteins to respective spots are given as additional files 1, 2 and 3 (Table S1, Figure S1, Dataset S1). Multiple (up to six) identifications in separate gels from individual secretory lysosomes preparations from T cells of different donors also underscore the reproducibility of the isolation protocol [11]. Overall, the identified spots represent a total of 397 separate protein entries in the NCBI database that are listed according to their protein names, the predicted subcellular distribution and function in Table 1. Importantly, based on database annotations combining proteome analyses of different organelles [17], 70% of the 397 proteins were assigned to lysosomal or secretory vesicles (including cytolytic granules (CG), lysosomes (LY), exosomes (EX), endosomes (EN), melanosomes (ME), platelet granules (PL) and synaptosomes (SY)) (Table 1, Figure 5). The majority of the remaining 30% was classified as proteins of unknown (11%) or cytosolic (11%) localization, and as cytosolic or nuclear proteins (CY/NU, 3.5%). The low percentage of mitochondrial (MT, 1.5%), nuclear (NU, 0.8%), plasma membrane (PM, 0.3%) or endoplasmic strictly reticulum-associated

Figure 4 2D proteome map of enriched secretory lysosomes from activated T cell blasts (overview). A total of 250 μg of fraction 2 protein were separated on pH 3-11NL IPG strips in the first and on 12.5% Tris-glycin gels in the second dimension. Proteins stained by Flamingo Pink were detected using fluorescence imaging. From a total of six gels, 742 spots were identified and annotated as 397 individual proteins. Enlarged sections of all four quadrants are available as additional file to allow the positioning of individual annotations given in table 1.
Table 1 Proteins identified in enriched secretory lysosomes from activated T cells

| Protein name                                                                 | Spot # | Predicted/annotated subcellular localisation | Predicted function          |
|------------------------------------------------------------------------------|--------|---------------------------------------------|----------------------------|
| 100 kDa coactivator                                                          | 122    | ER, ME, PL                                  | biosynthesis               |
| 14-3-3 protein beta                                                           | 714    | CY, ME                                      | adapter                    |
| 14-3-3 protein epsilon                                                        | 685    | CY, ME                                      | adapter                    |
| 14-3-3 protein zeta/delta                                                     | 708    | CY, ME                                      | adapter                    |
| 2',3'-cyclic-nucleotide 3'-phosphodiesterase                                 | 542    | ME, NG                                      | hydrolase                  |
| 26S protease regulatory subunit 6A                                            | 1037   | CY, NU                                      | protein degradation        |
| 26S proteosome non-ATPase regulatory subunit 2                                | 165    | ME                                          | proteasome                 |
| 3-phosphoglycerate dehydrogenase                                             | 408    | ME                                          | biosynthesis               |
| acetyl-CoA acetyltransferase, cytosolic                                       | 594    | CY                                          | biosynthesis               |
| ACTB protein                                                                 | 525    | ER, ME, PL, SY                             | cell motility              |
| actin related protein 2 isoform b                                            | 564    | EN, ER, ME, ME                             | trafficking                |
| actin related protein 2/3 complex subunit 1B                                 | 590    | ME, PL                                      | trafficking                |
| actin related protein 2/3 complex subunit 2                                  | 674    | EN, ME, PL, ER, ER                        | trafficking                |
| actin related protein 2/3 complex subunit 3                                  | 799    | EN, ME, PL, ER                            | trafficking                |
| actin related protein 2/3 complex subunit 4                                  | 798    | ER, EX, ME, SY                            | trafficking                |
| actin related protein 2/3 complex subunit S-like                             | 804    | ME                                          | trafficking                |
| actin, alpha, cardiac muscle                                                 | 570    | ME                                          | cell motility              |
| actin, gamma 1 propeptide                                                    | 701    | ME, EX, PL, SY                             | cell motility              |
| actinin, alpha 4                                                             | 144    | MENU, CY                                   | cell motility              |
| acylamino acid-releasing enzyme                                               | 203    | PL, CY                                      | hydrolase                  |
| acyl-CoA synthetase long-chain family member 4 isoform 2                     | 199    | MT, P, ME                                  | metabolism                |
| adenine phosphoribosyltransferase isoform b                                  | 784    | ME, EX, PL                                 | biosynthesis               |
| adenosine deaminase                                                          | 550    | CY, LY                                      | hydrolase                  |
| adenylsucinate lyase                                                          | 439    | CY, PL                                      | biosynthesis               |
| adenylsucinate synthetase                                                    | 1023   | CY                                          | biosynthesis               |
| adenylyl cyclase-associated protein variant                                  | 395    | PL, ME                                      | trafficking                |
| aflatoxin aldehyde reductase AFAR                                             | 1056   | PL, GO                                      | redox protein              |
| aging-associated gene 12                                                     | 418    | unknown                                    | unclassified               |
| alanyl-tRNA synthetase                                                        | 111    | ME, PL                                      | biosynthesis               |
| alcohol dehydrogenase class-3                                                | 587    | PL, CY                                      | redox protein              |
| aldo-keto reductase family 1, member A1                                       | 1078   | PL, SY                                      | metabolism                |
| aldolase A                                                                   | 560    | EN, ME                                      | metabolism                |
| aldose 1-epimerase (BLOCK25)                                                  | 596    | CY                                          | metabolism                |
| aldose reductase                                                             | 981    | ME, EX, MT                                 | metabolism                |
| alkylidihydroxyacetonephosphate synthase, peroxisomal                       | 273    | PE                                          | biosynthesis               |
| alpha-tubulin                                                                | 362    | PL                                          | cell motility              |
| annexin A1                                                                   | 615    | ME, MT                                      | trafficking                |
| annexin A11                                                                  | 415    | MT, EX, ME, secreted                       | exocytosis, signal trans. |
| annexin A2                                                                   | 631    | MT, EX, ME, secreted                       | exocytosis, signal trans. |
| annexin A4                                                                   | 668    | MT, EX, ME, SY, ER                        | trafficking                |
| annexin A5                                                                   | 660    | MT, EX, ME, ER                            | trafficking                |
| annexin A6                                                                   | 263    | MT, EX, ME, ER                            | trafficking                |
| annexin A7 isoform 2                                                          | 504    | EX, ME, PL                                 | exocytosis                 |
| ARP3 actin-related protein 3 homolog                                          | 465    | EN, ER, ME                                 | trafficking                |
| ARTS-1                                                                       | 136    | ER, ME                                      | immunity                   |
| aryl hydrocarbon receptor interacting protein                                 | 955    | unknown                                    | unclassified               |
| asparaginyl-tRNA synthetase                                                   | 306    | ME                                          | biosynthesis               |
| aspartate aminotransferase                                                    | 575    | CY                                          | biosynthesis               |
| ATP citrate lyase                                                            | 96     | ME, EX, PL                                 | biosynthesis               |
| ATP synthase, alpha subunit precursor                                         | 426    | ER, LY, NG, SY, MT                        | channel                    |

Schmidt et al. Cell Communication and Signaling 2011, 9:4
http://www.biosignaling.com/content/9/1/4
Page 5 of 16
| Protein Name                                                                 | Accession | Location(s)         | Function(s)          |
|------------------------------------------------------------------------------|-----------|---------------------|----------------------|
| ATPase, H+ transporting, lysosomal 56/S8kDa, V1 subunit B2                   | 413       | LY, MENG, SY        | channel              |
| axin interactor, dorsalization associated protein                            | 632       | unknown             | signal trans.        |
| beta adrenergic receptor kinase 1                                            | 220       | CY                  | GTPase               |
| bifunctional purine biosynthesis protein PURH                               | 326       | ME, PL              | multifunctional      |
| bleomycin hydrolase                                                         | 463       | CY, PL              | hydrolase            |
| Bola-like protein 2                                                         | 867       | ME                  | unclassified         |
| calcium binding protein 39                                                  | 622       | EX                  | unclassified         |
| CALM3 protein                                                                | 816       | unknown             | unclassified         |
| calreticulin precursor variant                                              | 287       | ER, MENG, EX, PL, MT| chaperone            |
| carboxyl terminal LIM domain protein                                         | 612       | ER, MEG, PL, EN     | cell motility        |
| catalase                                                                     | 361       | PE, E, L, E, M, E   | metabolism           |
| cathepsin B                                                                  | 691       | LY, MENG            | hydrolase            |
| cathepsin D preproprotein                                                    | 696       | LY, MENG, EX, MT    | hydrolase            |
| cathepsin H                                                                  | 738       | LY                  | hydrolase            |
| cathepsin S                                                                  | 749       | LY                  | immunity             |
| Cbr1 In Complex With Hydroxy-Pp                                               | 666       | ME                  | redox protein        |
| Cdc42ACK GTPASE                                                             | 790       | ME                  | cell motility        |
| centaurin beta1                                                              | 214       | unknown             | GTPase               |
| chaperonin (HSP60)                                                           | 338       | MENG, EX, SY, MT    | chaperone            |
| chaperonin containing TCP1, subunit 2β                                     | 424       | ERE, MENG, PL, MT, CY| chaperone            |
| chaperonin containing TCP1, subunit 7η                                      | 376       | EN, MEG, PL         | chaperone            |
| chaperonin containing TCP1, subunit 5ε                                      | 339       | EX, PL              | chaperone            |
| chaperonin containing TCP1, subunit 8τ                                      | 357       | EN, MEX              | chaperone            |
| chaperonin containing TCP1, subunit 8τ                                      | 358       | EN, MEX              | chaperone            |
| chromatin modifying protein 4B                                               | 639       | ME, EX              | trafficking           |
| chromosome 20 open reading frame 3 (BSCv)                                   | 489       | ME                  | unclassified         |
| chromosome 9 open reading frame 19                                           | 831       | EX, GO              | unclassified         |
| N2 protein                                                                  | 651       | unknown             | unclassified         |
| coactosin-like protein                                                       | 841       | PL, SY              | unclassified         |
| collin 1                                                                     | 810       | ERM, ME, EX, MT     | cell motility        |
| copine I                                                                    | 310       | ME                  | trafficking           |
| copine III                                                                  | 340       | ME, EX, PL          | trafficking           |
| coronin 7                                                                    | 113       | CY, GO              | trafficking           |
| coronin, actin binding protein, 1A                                            | 371       | LY, PL              | cell motility        |
| coronin, actin binding protein, 1C                                            | 353       | ME                  | multifunctional      |
| c-src tyrosine kinase                                                        | 475       | CY, PL              | signal trans.        |
| cyclophilin A                                                                | 826       | ME, EX, MT          | chaperone            |
| cyclophilin B                                                                | 800       | ERM, ME             | chaperone            |
| cystatin B                                                                  | 857       | ME                  | protein inhibitor    |
| cysteine and glycine-rich protein 1                                          | 769       | NU                  | unclassified         |
| cytoskeleton associated protein                                              | 952       | CY                  | cell motility        |
| cytosolic malate dehydrogenase                                               | 642       | ME, EX, PL, SY, MT  | metabolism           |
| DCHT2 Serine/threonine-protein kinase OSR1                                   | 332       | ME                  | signal trans.        |
| destin isoform a                                                             | 814       | ME, EX, MT          | cell motility        |
| differentially expressed in FDCP 6 homolog (mouse), isoform CRA_b           | 228       | unknown             | unclassified         |
| dihydropyrimidinase-like 2                                                  | 319       | SY                  | signal trans.        |
| dimethylarginine dimethylaminohydrolase                                      | 683       | unknown             | hydrolase            |
| dipeptidyl peptidase 4                                                       | 81        | ERL, L, E, M, E     | hydrolase            |
| DJ-1 protein                                                                | 764       | ME, PL, SY, MT      | redox protein        |
| DnaJ (Hsp40) homolog, subfamily A, member 1, isoform CRA_d                   | 499       | ERM, ME             | chaperone            |
| DnaJ (Hsp40) homolog, subfamily B, member 11 precursor                       | 551       | ER                  | chaperone            |
| docking protein 2                                                            | 455       | unknown             | unclassified         |
### Table 1: Proteins identified in enriched secretory lysosomes from activated T cells (Continued)

| Protein Name                                                                 | Gene ID | Expression Domain | Function |
|-----------------------------------------------------------------------------|---------|-------------------|----------|
| dynamin 2 isoform 1                                                         | 148     | EN                | trafficking |
| echinoderm microtubule associated protein like 2 variant                    | 929     | CY                | cell motility |
| EF-hand domain family, member D2                                             | 1012    | unknown           | unclassified |
| EH-domain containing 1                                                      | 342     | ERLY, EN, EX, PL  | trafficking |
| Ena-VASP-like protein                                                       | 447     | CY                | cell motility |
| ENC-1AS aka Beta-hexosaminidase subunit beta                                | 431     | LY                | multifunctional |
| endoplasmic reticulum protein 29 isoform 1 precursor                       | 721     | ER, ME, PL        | chaperone |
| enolase 1 variant                                                           | 496     | ME, EX, SY, MT   | metabolism |
| ERAP2 protein                                                               | 99      | ER                | immunity |
| ERB2IP protein                                                              | 197     | NU, CY            | multifunctional |
| ERO1L                                                                       | 311     | ME, ER            | redox protein |
| esterase D/formylglutathione hydrolase                                       | 656     | ME                | hydrolase |
| eukaryotic translation elongation factor 1 alpha 1                          | 462     | ERLY, EN, ME, EX, PL | biosynthesis |
| eukaryotic translation elongation factor 1 gamma, isoform CRA_d            | 947     | ME                | biosynthesis |
| eukaryotic translation elongation factor 2                                  | 158     | ER, EN, ME, EX    | biosynthesis |
| eukaryotic translation initiation factor 4A                                  | 505     | ME                | biosynthesis |
| extended-synaptotagmin-1 KIAA0747 protein                                    | 817     | ME                | biosynthesis |
| ezrin                                                                       | 208     | CY                | cell motility |
| F-actin capping protein alpha-1 subunit                                     | 611     | ERLY, ME          | actin binding |
| F-actin capping protein alpha-1 subunit variant                             | 623     | ERLY, ME          | actin binding |
| F-actin capping protein alpha-2 subunit                                     | 616     | ER, EN, ME, PL   | cell motility |
| F-actin capping protein beta subunit                                       | 663     | ER, EN, ME, PL   | actin binding |
| farnesyl pyrophosphate synthetase                                           | 579     | CY                | biosynthesis |
| FKS06 binding protein 1A                                                    | 856     | ME, SY, MT       | signal trans. |
| flotillin 1                                                                  | 486     | LY, ME, EX       | membrane |
| formin-binding protein 1                                                    | 1059    | SL, LY, CY       | adapter |
| fructose-bisphosphate aldolase C                                             | 565     | ME, SY, MT       | metabolism |
| fumarate hydratase, mitochondrial                                           | 507     | EN, SY, MT       | cell cycle |
| FYN-binding protein                                                         | 71      | CY, NU           | adapter |
| G protein beta subunit                                                      | 638     | ME, MT           | signal trans. |
| galectin-1                                                                  | 851     | ME, PL           | immunity |
| galectin-3                                                                  | 718     | ME, NU           | immunity |
| gamma-enolase                                                               | 476     | ME, PL, SY       | glycolysis |
| gamma-glutamyl hydrolase                                                    | 629     | LY, ME, EN, PL   | hydrolase |
| GDP-mannose pyrophosphorylase A                                             | 541     | unknown          | biosynthesis |
| gelatin-like capping protein isoform 9                                      | 572     | ME, CY, NU       | cell motility |
| GIPC1 protein                                                               | 598     | SY, CY           | protein binding |
| glia maturation factor gamma                                                 | 818     | unknown          | unclassified |
| glucosamine-6-phosphate deaminase 1                                        | 677     | CY                | hydrolase |
| glucose-6-phosphate dehydrogenase isoform b                                 | 409     | ME                | metabolism |
| glucosidase II subunit beta                                                 | 126     | ER, ME, PL       | hydrolase |
| glucosidase, alpha; neutral A8, isoform CRA_a                               | 936     | ER, ME, PL       | hydrolase |
| glutamate carboxypeptidase                                                   | 430     | unknown          | hydrolase |
| glutamate Dehydrogenase-Apo Form                                            | 437     | ERM, EP, MT      | unclassified |
| glutaredoxin 3                                                              | 589     | CY                | redox protein |
| glutathione S-transferase P1                                                | 766     | ERM, EP, MT      | metabolism |
| glutathione synthetase                                                      | 461     | PL                | redox protein |
| glutathione-S-transferase kappa 1                                           | 765     | PL, ME, MT, PE  | unclassified |
| glutathione-S-transferase omega 1                                           | 698     | LY, ME, NG, EP, PL, SY, MT | metabolism |
| glyceraldehyde-3-phosphate dehydrogenase                                   | 610     | LY, ME, NG, EP, PL, SY, MT | metabolism |
| glycyll-tRNA synthetase                                                     | 244     | ME                | biosynthesis |
Table 1  Proteins identified in enriched secretory lysosomes from activated T cells  (Continued)

| Protein                                                                 | Accession | Location       | Function          |
|------------------------------------------------------------------------|-----------|----------------|-------------------|
| glyoxalase domain containing 4                                         | 653       | MT             | unclassified      |
| GNAS complex locus isoform f                                           | 531       | EX             | multifunctional   |
| GNB1 protein                                                           | 634       | EN,ME,EX,PL,SY | signal trans.     |
| granzyme A                                                             | 724       | SL             | immunity          |
| GRAP2 protein                                                          | 957       | unknown        | unclassified      |
| GRB2 protein                                                           | 756       | SY             | adapter           |
| GTP-binding nuclear protein Ran                                        | 755       | ME,EX          | trafficking       |
| guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2, isoform CRA_c | 582       | EX             | GTPase            |
| guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1, isoform CRA_d | 664       | ER             | signal trans.     |
| guanine nucleotide-binding protein G(k) subunit alpha                  | 585       | ME,EX          | trafficking       |
| guanine nucleotide-binding protein subunit alpha-13                    | 989       | ME             | signal trans.     |
| haloacid dehalogenase-like hydrolase domain containing 2              | 690       | unknown        | hydrolase         |
| heat shock 70kDa protein 1A                                             | 278       | EN,ME,EX,MT    | chaperone         |
| heat shock 70kDa protein 5                                              | 226       | ER,ME,PL,MT    | chaperone         |
| heat shock 70kDa protein 8 isoform 1                                    | 259       | LY,ME,EN,EX,PL,SY,MT | chaperone |
| heat shock 70kDa protein 8 isoform 1                                    | 260       | LY,ME,EN,EX,PL,SY,MT | chaperone |
| heat shock protein 70                                                  | 112       | EX             | chaperone         |
| heat shock protein HSP 90-alpha                                         | 969       | ME,NG,MT      | chaperone         |
| heat shock protein HSP 90-beta                                         | 177       | ME,EX,MT      | chaperone         |
| hematopoietic cell-specific Lyn substrate 1                            | 181       | CY,MT          | signal trans.     |
| HEXA protein                                                           | 422       | LY             | multifunctional   |
| hexose-6-phosphate dehydrogenase                                       | 194       | ER             | metabolism        |
| HIP-55                                                                 | 377       | CY             | signal trans.     |
| histidine triad nucleotide binding protein 1                           | 852       | ME,PL,SY      | hydrolase         |
| histocompatibility (minor) HA-1                                        | 1072      | unknown        | GTPase            |
| hypothetical protein                                                   | 216       | unknown        | unclassified      |
| hypothetical protein LOC79624                                          | 472       | unknown        | unclassified      |
| hypoxia up-regulated protein 1                                          | 47        | ER,PL,ME      | chaperone         |
| importin subunit beta-1                                                | 164       | ME             | trafficking       |
| integrin beta-2                                                        | 80        | PL             | membrane          |
| interleukin-16                                                         | 210       | secreted       | immunity          |
| isocitrate dehydrogenase 1 (NADP+), soluble, isoform CRA_b             | 540       | ME,EX,PL      | redox protein     |
| isocitrate dehydrogenase 2 (NADP+), mitochondrial, isoform CRA_b       | 510       | PL,MT          | redox protein     |
| kinase/transmembrane domain fusion protein                             | 1061      | unknown        | unclassified      |
| laminin-binding protein                                                | 543       | ME,ER          | cell adhesion     |
| leucine aminopeptidase 3                                               | 432       | CY             | protein degradation |
| leucine rich repeat containing 5                                         | 747       | unknown        | unclassified      |
| leucine-rich repeat and calponin homology domain-containing protein 5  | 908       | MT             | protein binding   |
| leucocyte antigen CD97                                                  | 872       | ME,secreted    | cell adhesion     |
| leukocyte-derived arginine aminopeptidase long form variant            | 102       | unknown        | hydrolase         |
| leukotriene A4 hydrolase                                               | 309       | CY             | hydrolase         |
| LIM and SH3 domain protein 1                                            | 606       | EN,ME,PL      | adapter           |
| LIM domain-containing protein 2                                         | 834       | unknown        | unclassified      |
| lin 7 homolog c                                                        | 1070      | SY             | exocytosis        |
| L-lactate dehydrogenase                                                | 645       | ME,EX,SY      | metabolism        |
| L-lactate dehydrogenase B chain                                         | 626       | ME,EX,PL,SY,MT | redox protein     |
| L-plastin                                                              | 266       | CY             | actin binding     |
| L-plastin variant                                                      | 267       | unknown        | cell motility     |
| LPXN protein                                                           | 474       | unknown        | unclassified      |
| lymphocyte cytosolic protein 2                                         | 229       | CY             | immunity          |
| Protein Name | Kegg ID | Function |
|--------------|---------|----------|
| lymphocyte-specific protein 1 | 959 | PL | immunity |
| lysosomal acid alpha-mannosidase | 265 | LY,ME | hydrolase |
| M2-type pyruvate kinase | 356 | ME,EX,SY | metabolism |
| Macrophage Migration Inhibitory Factor (MIF) With Hydroxphenylpyruvate | 862 | ME,EX,PL,SY | immunity |
| MAGUK p55 subfamily member 7 | 292 | PL | protein binding |
| methylenetetrahydrofolate dehydrogenase 1 | 139 | EN,ME,PL,MT | multifunctional |
| methylthioadenosine phosphorylase | 697 | CY | metabolism |
| MHC class I antigen | 533 | ME | immunity |
| MHC class I antigen | 865 | ME | immunity |
| MHC class II antigen | 953 | ME, EX | immunity |
| MHC class II antigen DR alpha chain | 1050 | LY | immunity |
| MHC class II antigen DR52 | 1083 | ME | immunity |
| microtubule-associated protein, RP/EB family, member 1 | 665 | MEPL | cell motility |
| mitochondrial ATP synthase, H+ transporting F1 complex beta subunit | 443 | MT | trafficking |
| mitochondrial trifunctional protein, alpha subunit precursor | 253 | PL, MT | metabolism |
| mitogen-activated protein kinase 1 | 569 | MEPL | signal trans. |
| mitogen-activated protein kinase kinase 1 interacting protein 1 | 943 | LY | adapter |
| mitogen-activated protein kinase kinase 2 | 509 | unknown | signal trans. |
| moesin, isoform CRA_b | 246 | EN,ME,EX,PL,MT | cell motility |
| mps one binder kinase activator-like 1B | 758 | unknown | unclassified |
| myosin IG | 75 | unknown | trafficking |
| myosin light polypeptide 6 | 830 | ME | cell motility |
| NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NADH-coenzyme Q reductase) | 896 | ER,ME,MT | trafficking |
| NCK adaptor protein 1 | 506 | CY,ER | adapter |
| NECAP endocytosis associated 2 | 1010 | EN | trafficking |
| NESH protein | 434 | unknown | unclassified |
| N-ethylmaleimide-sensitive factor attachment protein, alpha | 652 | ME,NG,PL | trafficking |
| neuroblastoma RAS viral (v-ras) oncogene homolog | 779 | GO,CY | trafficking |
| neuropolyptide h3 | 781 | ME,EX,SY | protein inhibitor |
| neutrophil adherence receptor alpha-M subunit | 36 | membrane | cell adhesion |
| ribon protein isoform 2 | 38 | CY | signal trans. |
| NME1-NME2 protein | 823 | CY,NU | multifunctional |
| nuclear chloride channel | 684 | ME,EX,PL,MT | channel |
| nucleobindin 1 variant | 335 | unknown | unclassified |
| nucleoside phosphorylase | 670 | CY,PL | cell cycle |
| nucleosome assembly protein 1-like 1, isoform CRA_d | 315 | ME,PL,NU | cell cycle |
| Obg-like ATPase 1 | 511 | EN,ME,PL | hydrolase |
| otubain 1 | 637 | ME | hydrolase |
| PA2G4 protein | 490 | unknown | unclassified |
| PDCD6IP protein | 171 | unknown | unclassified |
| perforin-1 | 280 | SL | immunity |
| peroxiredoxin 1 | 774 | ERLY,EN,ME,NG,PL,MT | redox protein |
| peroxiredoxin 2 | 778 | EN,ME,SY,MT | redox protein |
| peroxiredoxin 3 | 768 | ME,PL,MT | redox protein |
| peroxiredoxin 4 | 737 | EREN,ME | redox protein |
| peroxiredoxin 6 | 945 | LY,ME,EX,PL,SY | redox protein |
| PGAM1 | 730 | ME,EX,SY | metabolism |
| PHB | 948 | unknown | unclassified |
| phosphatase 2a | 316 | MT | multifunctional |
| phosphatidylinositol-5-phosphate 4-kinase, type II, alpha | 457 | NGPL | metabolism |
| phosphofructokinase, liver | 939 | unknown | glycolysis |
| Protein Name                                                                 | Frequency | Location | Function               |
|----------------------------------------------------------------------------|-----------|----------|------------------------|
| Phosphofructokinase, platelet                                             | 196       | ME, PL   | Glycolysis             |
| Phosphoglucose isomerase                                                  | 390       | ME, EX, PL, MT | Multifunctional       |
| Phosphoglycerate kinase 1                                                 | 537       | ME, EX, SY, MT | Metabolism           |
| Phospholipase C, delta 1 variant                                          | 178       | Unknown  | Signal trans.          |
| Phosphoribosyl pyrophosphate synthetase 1 variant                        | 1080      | Unknown  | Biosynthesis           |
| Phosphoribosylaminomimidazole carboxylase, - succinocarboxamide synthetase, isoform CRA_b | 523       | EN, SY   | Multifunctional        |
| Phosphoribosylformylglycinamidine synthase                                | 46       | CY       | Biosynthesis           |
| Phosphoserine aminotransferase 1                                          | 988       | ME       | Biosynthesis           |
| Phostensin                                                                | 91        | CY       | Unclassified           |
| Poly(A) binding protein, cytoplasmic 1, isoform CRA_c                     | 256       | ER, EN, ME, PL | Metabolism            |
| Poly(C) binding protein 1                                                 | 1082      | ME, CY, NU | Unclassified          |
| Potassium voltage-gated channel, shaker-related subfamily, beta member 2 isoform 2 | 635 | CY | Channel |
| PPPSC protein                                                             | 364       | CY, NU   | Hydrolase              |
| Profilin-1                                                                | 848       | ME, EX, PL, MT | Actin binding         |
| Programmed cell death protein 10                                           | 741       | Unknown  | Apoptosis              |
| Proline synthetase co-transcribed homolog                                 | 699       | CY       | Unclassified           |
| Prolyl 4-hydroxylyase, alpha subunit                                      | 337       | ER, ME   | Redox protein          |
| Prolyl 4-hydroxylyase, beta subunit precursor                             | 348       | ER, ME, EX, PL, MT | Redox protein     |
| Prolyl endopeptidase                                                      | 234       | CY       | Protein degradation    |
| Proteasome (prosome, macropain) subunit, alpha type, 7(PSMA7)             | 729       | CY, Proteasome | Hydrolase           |
| Proteasome 26S non-ATPase subunit 13 isoform 1                             | 577       | ME       | Proteasome             |
| Proteasome 26S subunit, ATPase, 2                                          | 498       | CY, NU   | Unclassified           |
| Proteasome 26S subunit, ATPase, 5                                          | 514       | CY, NU   | Unclassified           |
| Proteasome activator complex subunit 1 isoform 1                           | 703       | PL, MT   | Immunity               |
| Proteasome activator complex subunit 2                                    | 689       | ME       | Immunity               |
| Proteasome alpha 2 subunit variant                                        | 754       | CY       | Hydrolase              |
| Proteasome subunit, alpha type, 1                                          | 687       | ME       | Hydrolase              |
| Proteasome subunit, alpha type, 5                                          | 1009      | ME       | Hydrolase              |
| Proteasome subunit, alpha type, 6                                          | 734       | CY, NU   | Hydrolase              |
| Proteasome subunit, beta type, 1                                           | 750       | ME, CY   | Hydrolase              |
| Proteasome subunit, beta type, 2                                           | 780       | CY, NU   | Hydrolase              |
| Proteasome subunit, beta type, 4                                           | 944       | CY, NU   | Hydrolase              |
| Proteasome subunit, beta type, 8                                           | 773       | PL, CY, NU | Immunity            |
| Protein ARMET                                                             | 805       | ME, secreted | Unclassified     |
| Protein diaphanous homolog 1                                              | 45        | ME       | Cell motility          |
| Protein disulfide isomerase-associated 4                                   | 1060      | ER, ME, PL | Chaperone            |
| Protein disulfide isomerase-related protein 5                              | 458       | ER, ME   | Chaperone              |
| Protein disulfide-isomerase A3                                             | 379       | ER, LY, ME, NG, EX, PL | Chaperone    |
| Protein phosphatase 1, catalytic subunit, alpha isoform 1                 | 603       | EX       | Hydrolase              |
| Protein phosphatase 1, catalytic subunit, beta isoform                     | 617       | ME, PL   | Hydrolase              |
| Protein tyrosine phosphatase 1b                                            | 536       | ME, ER   | Hydrolase              |
| Protein tyrosine phosphatase, non-receptor type 6 isoform 1 variant       | 317       | Unknown  | Hydrolase              |
| Protein-tyrosine kinase fyn isoform c                                      | 373       | EN, CY   | Signal trans.          |
| PYD and CARD domain containing                                            | 771       | CY       | Apoptosis              |
| Pyrophosphatase 1                                                          | 654       | ME, MT   | Hydrolase              |
| Pyruvate kinase 3 isoform 2                                                | 346       | ME, EX, SY | Metabolism           |
| R33729_1 (Interleukin-25)                                                 | 837       | ME, secreted | Signal trans. |
| Rab GDP dissociation inhibitor beta                                        | 469       | ME, EX, PL, MT | GTPase    |
| Raf1 cell migration-inducing gene 2                                        | 193       | PL       | Unclassified           |
| Rap1a                                                                     | 785       | EN, ME, MT | GTPase                 |
Table 1 Proteins identified in enriched secretory lysosomes from activated T cells (Continued)

| Protein Description                                                                 | Accession | Location | Function |
|-------------------------------------------------------------------------------------|-----------|----------|----------|
| Rap1-GTP-interacting adapter molecule                                               | 141       | CY       | signal trans. |
| Ras GTPase-activating-like protein IQGAP2                                            | 1069a     | EN       | signal trans. |
| related RAS viral (t-ras) oncogene homolog 2 isoform a                              | 1049      | LY,ME,EX | GTPase    |
| Rho GTPase activating protein 1                                                      | 716       | ME,PL,MT | GTPase    |
| Rho GTPase activating protein 9                                                      | 728       | CY       | GTPase    |
| ribosomal protein L11                                                                 | 797       | ENribose | biosynthesis |
| ribosomal protein L12                                                                 | 809       | MEribose | biosynthesis |
| S-adenosylhomocysteine hydrolase                                                    | 513       | ME       | hydrolase |
| Sec23 homolog A                                                                      | 221       | ER,ME,PL | trafficking |
| Sec23B protein                                                                       | 1001      | EN       | trafficking |
| septin 2                                                                            | 554       | ME,EX,SY | unclassified |
| septin 7                                                                            | 484       | ME,PL,SY | unclassified |
| septin-9 delta                                                                       | 558       | ME       | unclassified |
| septin-9 gamma                                                                       | 973       | ME       | unclassified |
| serine/threonine phosphatase 1 gamma                                                | 985       | MT,SY    | hydrolase |
| serine/threonine-protein kinase PAK 2                                                | 352       | PL       | signal trans. |
| serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform             | 621       | MT       | signal trans. |
| serine/threonine-protein phosphatase 2A regulatory subunit B                        | 592       | NU       | signal trans. |
| serpin peptidase inhibitor, clade B, member 1                                       | 548       | CY       | protein inhibitor |
| SH2 domain protein 1A                                                                 | 840       | CY       | signal trans. |
| SH3-containing protein, Endophilin-B1                                                | 1081      | CY,GO,MT | apoptosis |
| SHUJUN-1                                                                            | 795       | CY       | cell motility |
| similar to metallo-beta-lactamase superfamily protein                               | 188       | CY,NU    | signal trans. |
| small GTP binding protein Rac2, isoform CRA_c                                       | 1006      | unknown  | hydrolyase |
| soc-2 suppressor of clear homolog                                                   | 318       | CY       | unclassified |
| solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1          | 460       | ME,EX,PL | scaffolding |
| sorting nexin 17                                                                    | 421       | EN,ME,PL | trafficking |
| sorting nexin 6                                                                     | 466       | CY       | trafficking |
| src kinase associated phosphoprotein 1 isoform 1                                    | 417       | CY,NU    | signal trans. |
| stathmin 1/oncoprotein 18                                                            | 820       | SY       | cell motility |
| stress-induced phosphoprotein 1 (Hsp70/Hsp90-organizing protein)                    | 323a      | ME,PL,SY | chaperone |
| stromal cell-derived factor 2-like 1 precursor                                       | 767       | ER       | unclassified |
| superoxide dismutase 1, soluble                                                     | 806       | ME,EX,MT | redox protein |
| syntaxin binding protein 1                                                           | 305       | MENG,EX,PLSY | trafficking |
| syntaxin binding protein 2                                                           | 302       | EX,PL    | trafficking |
| syntaxin binding protein 3 variant                                                   | 294       | ME,PL    | trafficking |
| talin-1                                                                             | 920       | EN,ME,PL | cell motility |
| tapasin isoform 3 precursor                                                          | 495       | ER,ME    | immunity |
| TC4 protein                                                                         | 736       | NU       | GTPase    |
| T-complex polypeptide 1                                                               | 1030      | ER,EN,ME,EX | chaperone |
| T-complex protein 1 subunit gamma                                                     | 307       | CY       | chaperone |
| testin isoform 1                                                                     | 456       | unknown  | unclassified |
| thioredoxin domain-containing protein 4 precursor                                    | 502       | ER,ME,PL | scaffolding |
| transfer RNA-Trp synthetase                                                          | 411       | ME,PL    | biosynthesis |
| transgulin-2                                                                         | 787       | ME,MT    | unclassified |
| transketolase                                                                       | 276       | ME,NEN   | unclassified |
| translocon-associated protein subunit delta                                           | 811       | MEER     | trafficking |
Table 1 Proteins identified in enriched secretory lysosomes from activated T cells (Continued)

| Protein Name | Spot Number | Subcellular Localisation | Function |
|--------------|-------------|--------------------------|----------|
| Triosephosphate isomerase 1 | 742 | ME, EX, SY, MT | unclassified |
| Tripeptidyl-peptidase 1 | 992 | LY, ME, NG, PL, MT | protein degradation |
| Tropomodulin 3 | 561 | ER, ME | cell motility |
| Tropomyosin 3 isoform 2 | 676 | unknown | unclassified |
| Tropomyosin 4 | 672 | ME | unclassified |
| Tu translation elongation factor, mitochondrial | 517 | LY, ME, PL, MT | biosynthesis |
| Tubulin alpha 6 variant | 363 | ME, PL | cell motility |
| Tubulin tyrosine ligase-like family, member 12 | 897 | ME | trafficking |
| Tubulin, beta | 407 | ME, PL, SY | cell motility |
| Tubulin, beta polypeptide | 433 | ME, PL, SY | cell motility |
| Tumor rejection antigen (gp96) 1 | 118 | ER, ME, PL, GO | chaperone |
| Tumor susceptibility gene 101 | 470 | EX | trafficking |
| Twinfilin-like protein | 578 | CY | cell motility |
| Tyrosine kinase LCK | 399 | CY | signal trans. |
| Tyrosine-protein phosphatase non-receptor type 6 | 325 | CY, NU | signal trans. |
| Tyrosyl-RNA synthetase | 366 | ME, PL | signal trans. |
| Ubiquitin associated and SH3 domain containing protein A | 913 | CY, NU | protein degradation |
| Ubiquitin specific peptidase 5 isoform 2 | 154 | LY, ME, NG | protein degradation |
| Ubiquitin specific peptidase 14 isoform a | 344 | PL | protein degradation |
| Ubiquitin-conjugating enzyme E2 L3 | 882 | MT | protein degradation |
| Ubiquitin-conjugating enzyme E2 N | 839 | ME, EX, MT | differentiation |
| Ubiquitin-like modifier-activating enzyme 1 | 120 | MT, ME | protein degradation |
| UDP-glucose ceramide glucosyltransferase-like 1 isoform 1 | 39 | ER, ME | chaperone |
| UDP-glucose pyrophosphorylase 2 isoform b | 442 | EN, ME | metabolism |
| UNC-112 related protein 2 long form | 971 | PL | cell adhesion |
| Unnamed protein product | 706 | unknown | unclassified |
| UPF0550 protein C7orf28 | 450 | ME | unclassified |
| Vacuolar H+-ATPase 56,000 subunit | 414 | LY, ME, GSY | channel |
| Vacuolar protein sorting 45A | 322 | LY, EN | trafficking |
| Vacuolar sorting protein 33A | 1067 | EN, LY | trafficking |
| Valosin-containing protein | 159 | unknown | unclassified |
| Vasodilator-stimulated phosphoprotein | 503 | PL | cell motility |
| Vinculin | 108 | ME | cell motility |
| Voltage-dependent anion channel 1 | 658 | ERLY, ME, G, EX, PL, SY, MT | channel |
| Voltage-dependent anion channel 2 | 657 | SY, MT | channel |
| Voltage-dependent anion channel 3 | 688 | EN, ME, MT | channel |
| V-type proton ATPase subunit d 1 | 599 | LY, EN, ME, SY | channel |
| WD repeat domain 1 | 304 | EN, ME, EX | cell motility |
| Wiskott-Aldrich syndrome protein | 323b | CY | cell motility |
| XRP2 protein | 546 | ME | signal trans. |
| Zeta-chain associated protein kinase 70kDa | 277 | CY | signal trans. |

397 individual proteins were identified to be associated with enriched secretory lysosomes from human T cell blasts. The proteins are listed by name, followed by individual spot numbers and the predicted/annotated subcellular localisation and function. Abbreviations: LY: lysosomes, ME: melanosomes, PL: platelet granules, SY: synaptosomes, EX: exosomes, CG: cytotoxic granules, NG: neuromelanin granules, EN: endosomes, MT: mitochondria, GO: Golgi, PE: peroxisomes, CY: cytoplasm, ER: endoplasmic reticulum and NU: nucleus. For detailed information on individual spots/proteins, please refer to the additional files.
(ER, 1.5%) and peroxisomal proteins (PE, 0.3%) again underscores the selective enrichment of lysosomal organelles in the present study. In terms of function, the classification revealed a large heterogeneity and a broad spectrum of potential activities. However, as expected, proteins associated with degradation, signal transduction, trafficking and immunity formed about 35% of the total proteome of enriched SL (Figure 5B). The important role of these organelles in cytotoxicity is also supported by the identified effector molecules perforin (#280) and granzyme A (#707, 717, 720, 724).

Interestingly, and in contrast to the published SL proteome of NK cells [12], we did not detect significant amounts of granzyme B at the respective position in 2D gels from T cell blasts. However, this is in line with our previous observation that granzyme B might be stored in a separate compartment formed by electron dense granules that do not contain transmembrane FasL and

---

**Figure 5** Classification of the identified proteins according to their localization (A) and function (B). The cellular localization and function of 397 identified proteins were classified as detailed in material and methods. Lysosomal and secretory vesicles are represented by melanosomes (ME), lysosomes (LY), platelet granules (PL), endosomes (EN), synaptosomes (SY), exosomes (EX) or cytolytic granules (CG). Other cellular compartments are: cytosol (CY), nuclei (NU), peroxisomes (PE), plasma membrane (PM), mitochondria (MT), golgi (GO) or endoplasmic reticulum (ER).
that sediment as fraction 6 in our enrichment gradient [11]. To prove this result and address this issue in more detail, we started to analyze fraction 2 and fraction 6 vesicles (granzyme B granules). The direct comparison of the two granule populations by 2D DIGE and Western blotting clearly verified the result of the present analysis and provided first biochemical and proteomic evidence for two distinct species of cytotoxic effector vesicles in T cell blasts [18].

Surprisingly, it is still unknown to date whether functionally distinct TCRαβ and TCRγδ T cells, CD4+ and CD8+ T cells, Vδ1+ and Vδ2+ T cells, or normal and leukemic T cells also differ in terms of protein content and function of their lysosomal compartment(s). Based on the present description of the luminal proteome of FasL-containing secretory lysosomes in fully differentiated T cells, it will be possible to directly compare the content of cytotoxic effector organelles in different T cell subpopulations, e.g. by 2D difference gel electrophoresis. In addition, based on a larger set of marker proteins, the maturation of effector vesicles in the course of T cell activation can now be addressed in detail. Of note, using the applied protocol, we identified the luminal rather than the membrane proteome of this vesicular population. In addition, one has to consider that due to methodological limitations, the applied 2D technique might cover only about 20-30% of the total proteome and thus might be complemented in future studies employing LC-coupled mass spectrometric approaches.

**Conclusion**
We provide the first comprehensive proteome map of T cell-derived secretory lysosomes with only minor contaminations by cytosolic, nuclear or other proteins. This information will be useful to more precisely address the activation-dependent maturation and the specific distribution of effector organelles and proteins in individual T or NK cell populations in future studies.

**Methods**

**Cells**
Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat preparations by Ficoll density gradient centrifugation. For the generation of PHA-stimulated lymphoblasts, T cells were purified by magnetic cell sorting (MACS) using cell isolation kits from Miltenyi Biotech (Bergisch Gladbach, Germany). The cells were stimulated with phytohemagglutinin A (PHA, 0.5 µg/ml, Remel, Lenexa, KS, USA) and expanded in the presence of irradiated EBV-transformed B cells and allogenic PBMC and subsequently with recombinant interleukin 2 (rIL-2, 100 U/ml, Chiron GmbH, Marburg, Germany). Before the cells were analyzed on day 12-14, dead cells were removed by Ficoll-gradient centrifugation resulting in a > 98% pure T cell population as judged by CD3 FACS analysis.

**Confocal microscopy**
Cells were fixed with 3% paraformaldehyde and permeabilized with 1% Triton X-100 as described [13]. The following antibodies were used: mouse IgG1 isotype control MOPC-21 (Abcam, Cambridge, UK), anti-FasL mAb NOK1 (BD Biosciences, Heidelberg, Germany) with AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany), anti-CD63 mAb clone MEM-259 (Immunotools, Friesoythe, Germany) conjugated to AlexaFluor555 (Invitrogen), anti-Granzyme A-FITC (Immunotools) and anti-Cathepsin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with AlexaFluor488-conjugated donkey anti-goat IgG (Invitrogen). Stained samples were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and analyzed on a laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) with appropriate filter settings. Images were acquired via scanning through the x-y-plane with 63 × objective lense. Laser intensity and detectors were adjusted to a uniformly negative signal of the control samples stained with control IgG and second step antibodies.

**Subcellular fractionation**
For subcellular fractionation and enrichment of secretory lysosomes, at least 4x10^8 T cells were used. The fractionation procedure has been recently described in detail [11]. Briefly, the cells were mechanically disrupted and organelles were enriched by differential centrifugation steps. The enriched organelles were then loaded on a discontinuous density gradient (4.4 ml volume) with 27%, 22.5%, 19%, 16%, 12%, 8% Optiprep® which is a 60% Iodixanol solution (Sigma, Deisenhofen, Germany) and subjected to ultracentrifugation. Interphases were collected from the top of the gradient resulting in six 400 µl fractions named and numbered 1 to 6. The protein content in each fraction was determined using a Coomassie Protein Assay Reagent (Thermo, Rockford, IL, USA).

**Western blot analysis**
For Western blotting, 5 µg of protein were separated by SDS-PAGE on pre-cast endothelial antibody control MOPS-21 (Abcam, Cambridge, UK), anti-FasL mAb NOK1 (BD Biosciences, Heidelberg, Germany) with AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany), anti-CD63 mAb clone MEM-259 (Acris Antibodies, Herford, Germany), anti-LAMP-1 clone MEM-259 (BD Biosciences, Heidelberg, Germany), anti-CD63 clone MEM-259 (Acris Antibodies, Herford, Germany), anti-LAMP-1 clone 25 (BD Biosciences), anti-FasL clone G-247.4 (BD Biosciences, Heidelberg, Germany) and subjected to ultracentrifugation. Interphases were collected from the top of the gradient resulting in six 400 µl fractions named and numbered 1 to 6. The protein content in each fraction was determined using a Coomassie Protein Assay Reagent (Thermo, Rockford, IL, USA).
anti-cathepsin D clone CTD-19 (Sigma), anti-cytochrome oxidase IV (CoxIV) mAb clone 10G8D12C12 (1/1000, MitoScience, Eugene, OR, USA), anti pan-cadherin clone ab22744 (Abcam, Cambridge, UK) and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (GE Healthcare, Munich, Germany). Membranes were prepared for reprobing by incubation in stripping solution (100 mM 2-mercaptoethanol, 2% SDS, 60 mM Tris) for 25 min at 56°C. ECL reagents in combination with Hyper Film (GE Healthcare) were used for chemiluminescence detection.

Transmission electron microscopy
Enriched organelles of fractions 2 and 5 were fixed with a mixture of 3% paraformaldehyde and 0.05% glutaraldehyde in PBS at 4°C overnight, washed in PBS, postfixed in 2% OsO₄ dehydrated in ethanol, and embedded in araldite (Sigma, Deisenhofen, Germany). Ultrathin sections were mounted on formvar-coated grids and double-stained with a saturated solution of uranyl acetate in 70% methanol and lead citrate. The grids were examined with a Zeiss EM 900 transmission electron microscope equipped with a digital camera system.

2D electrophoresis, image analysis and spot picking
The 2D electrophoresis was performed as described before [11]. Briefly, SL pellets of fraction 2 were lysed on ice for 30 min with 30 μl lysis buffer (pH 8.5) containing 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS. The supernatant was recovered after centrifugation for 20 min at 20,000 × g at 4°C. A total amount of 250 μg of protein was mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% (v/v) IPG buffer pH 3-11 and 2% (w/v) DTT) and applied by cup-loading onto 24 cm non-linear pH 3-11 IPG gel strips (7 M urea, 2 M thiourea, 4% CHAPS, 2% (v/v), IPG buffer 7 M urea, 2 M thiourea, 4% CHAPS, 2% (w/v) IPG buffer pH 3-11 and 2% (w/v) DTT) and applied by cup-loading onto 24 cm non-linear pH 3-11 IPG gel strips for isoelectric focusing (IEF). The second dimension was performed on 26 × 20 cm large 12.5% polyacrylamide gels after reduction and alkylation using the Ettan DALTsix large vertical electrophoresis system from GE Healthcare. The gels were removed from the glass plates, stained with Flamingo Pink (Bio Rad), mounted on a non-backed gel frame, scanned on a Typhoon Trio imager (GE Healthcare) and analyzed using Image Master 6.0 (GE Healthcare). Selected spots were picked with a 2 mm picking head. The picked gels were again scanned to verify the correct location of the punched spots.

In-gel tryptic digestion and mass spectrometry
Gel plugs were washed with water and 12.5 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) and dehydrated in pure ACN. The dry gel pieces were rehydrated with 100 ng sequencing-grade trypsin (Serva, Heidelberg, Germany) in 5 mM ABC and tryptic in-gel digestion was performed at 37°C overnight. For peptide extraction, 0.3% trifluoroacetic acid (TFA) in ACN was added and the samples were sonicated for 15 min. The liquid phases were collected, lyophilized, redissolved in 0.5 to 1 μl MALDI matrix solution (3.2 mg/ml α-cyanohydroxycinnamic acid (Sigma) in 65% ACN/0.1% TFA), spotted onto 192-well stainless steel MALDI plates and air-dried. The samples were analyzed by peptide mass finger printing in positive reflector mode followed by MSMS analyses of the most apparent five peptides using the 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA, USA) as described elsewhere [12]. Peptide mass spectra were processed by internal calibration with autolytic fragments of porcine trypsin with 25 ppm mass tolerance. MSMS spectra were acquired using default calibration updated prior to the run. Spectral data were searched against human proteins in the NCBI database (Homo sapiens, 192,176 entries) using MASCOT V2.0 (Matrix Sciences, London, UK).

Database analysis
Database searches with MASCOT were performed using the following parameters: the modification on cysteine residues by carbamidomethylation was set as obligate, methionine oxidation was considered as a potential modification; the maximum number of missed tryptic cleavages was one; the monoisotopic masses were considered and the mass tolerance was set to ± 50 ppm, and the fragment-ion mass tolerance was set to 0.2 Da (MS/MS). A protein was accepted to be identified when the total protein score reached or exceeded the MASCOT score threshold (≥ 65 with a probability of identification greater 95%). A repeated search against a randomized decoy database (http://www.matrixscience.com/help/decoy_help.html) using the decoy.pl script and identical search parameters let to a false-positive rate of 1.2%.

The classification according the localization and function of individual proteins was based on the UniProt knowledge base, the iHOP database [19] and the iProXpress database [17] available through the Protein Information Resource (PIR) (GUMC, Washington DC, USA). Identified proteins were searched in this organelle-proteome reference dataset according to their UniProt numbers.

Additional material

Additional file 1: Table S1. List of identified spots in enriched SL preparations from activated T cells. 742 spots representing 397 proteins were identified and annotated according to Figure S1 A-D. Proteins (3) are listed with spot numbers (1), the number of identifications (2), respective NCBI accession (4) and UniProt (5) numbers, theoretical molecular weights (MW) (6) and isoelectric points.
8. Bossi G, Griffiths GM: Degranulation plays an essential part in regulating secretory lysosomes and their expression and function of.

5. Pols MS, Klumperman J: Trafficking and function of the tetraspanin CD63.

2. Bright NA, Reaves BJ, Mullock BM, Luzio JP: A receptor for the selective uptake and degradation of dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. J Cell Sci 1997, 110:2027-2040.

Author details
1 Institute of Immunology, Christian-Albrechts-University, UK S-H Campus Kiel, Kiel, Germany. 2 Department of Zoophysiology, Zoological Institute, Christian-Albrechts-University, Kiel, Germany. 3 Institute of Anatomy, Christian-Albrechts-University, Kiel, Germany.

Authors’ contributions
HS and MN performed all experiments regarding cell culture, lysosome enrichment and 2D gel electrophoresis. MLet performed the confocal imaging experiments and was involved in the establishment of the lysosomal purification protocol. RL performed the electron microscopy. CG carried out all mass spectrometrical analyses. HS, MN and CG performed data analyses and assignments. HS, MLei, DK and OJ conceived of the study, and participated in its design and coordination. HS, CG and OJ drafted the manuscript. All authors read and approved the manuscript.

Competing interests
All authors declare that they have no competing interests.

Received: 1 December 2010 Accepted: 21 January 2011

References
1. Blott EJ, Griffiths GM: Secretory lysosomes. Nat Rev Mol Cell Biol 2002, 3:122-131.

2. Bright NA, Reaves BJ, Mullock BM, Luzio JP: Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. J Cell Sci 1997, 110:2027-2040.

3. Carlson SR, Fukuda M: Structure of human lysosomal membrane glycoprotein 1: Assignment of disulfide bonds and visualization of its domain arrangement. J Biol Chem 1989, 264:20526-20531.

4. Cuervo AM, Dice JF: A receptor for the selective uptake and degradation of proteins by lysosomes. Science 1996, 273:501-503.

5. Pols MS, Klumperman J: Trafficking and function of the tetraspanin CD63. Exp Cell Res 2009, 315:1584-92.

6. Fowler KT, Andrews NW, Huleatt JW: Expression and function of synaptotagmin VII in CTLs. J Immunol 2007, 178:1486-1504.

7. Lettau M, Schmidt H, Kabelitz D, Janssen O: Secretory lysosomes and their cargo in T and NK cells. Immunol Lett 2007, 108:10-19.

8. Bossi G, Griffiths GM: Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. Nat Med 1995, 1:90-96.

9. Blott EJ, Bossi G, Clark R, Zvelebil M, Griffiths GM: Fas ligand is targeted to secretory lysosomes via a proline-rich domain in its cytoplasmic tail. J Cell Sci 2001, 114:2405-2416.

10. Voss M, Lettau M, Paulsen M, Janssen O: Posttranslational regulation of Fas ligand function. Cell Commun Signal 2008, 6:11.

11. Schmidt H, Gelhaus C, Luquis R, Nebendahl M, Leippe M, Janssen O: Enrichment and analysis of secretory lysosomes from lymphocyte populations. BMC Immunol 2009, 10:41.

12. Schmidt H, Gelhaus C, Nebendahl M, Lettau M, Watzf, Kabelitz D, Leippe M, Janssen O: The adaptor protein Nck interacts with Fas ligand: Guiding the death factor to the cytotoxic immunological synapse. Proc Natl Acad Sci USA 2008, 105:5911-5916.

13. Lettau M, Qian J, Linkermann A, Latrelle M, Larose L, Kabelitz D, Janssen O: Regulation of FasL expression: a SH3 domain containing protein family involved in the lysosomal association of FasL. Cell Signal 2006, 18:1327-1337.

14. Lettau M, Pieper J, Janssen O: Nck proteins: Functional versatility in T cells. Cell Commun Signal 2009, 7:1.

15. Qian J, Chen W, Lettau M, Podda G, Zornig M, Kabelitz D, Janssen O: Regulation of FasL expression: a SH3 domain containing protein family involved in the lysosomal association of Fasl. Cell Signal 2006, 18:1327-1337.

16. Kassahn D, Nachbur U, Conus S, Micheau O, Schneider P, Simon HU, Brunner T: Distinct requirements for activation-induced cell surface expression of preformed Fas/CD95 ligand and cytoplasmic granule markers in T cells. Cell Death Differ 2009, 16:115-124.

17. Hu ZZ, Valencia JC, Huang H, Chi A, Shabanowitz J, Hearing VJ, Appella E, Wu C: Comparative Bioinformatics Analyses and Profiling of Lysosome-Related Organelle Proteomes. Int J Mass Spectrom 2007, 259:147-160.

18. Schmitz H, Gelhaus C, Nebendahl M, Lettau M, Luquis R, Leippe M, Kabelitz D, Janssen O: Effector granules in human T lymphocytes: Proteomic evidence for two distinct species of cytotoxic effector vesicles. J Proteome Res 2011, [Epub ahead of print].

19. Hoffmann R, Valencia A: A gene network for navigating the literature. Nat Genet 2004, 36:664.

Cite this article as: Schmidt et al.: Effector granules in human T lymphocytes: the luminal proteome of secretory lysosomes from human T cells. Cell Communication and Signaling 2011 9:4.

doi:10.1186/1478-811X-9-4