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Compensatory increase in USP14 activity accompanies impaired proteasomal proteolysis during aging.

Subramaniam Ponnappan^a, Michela Palmieri^b, Dennis H. Sullivan^{a,c}, and Usha Ponnappan^{a,b,*}

^a Department of Geriatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA.

^b Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA.

^c Geriatric Research Education and Clinical Center, Central Arkansas Veteran's Healthcare System (3J/NLR), North Little Rock, AR 72114, USA.

Abstract

The deubiquitinating enzyme, USP14, found in association with the proteasome is essential in mediating ubiquitin trimming and in ensuring ubiquitin-homeostasis. As aging is accompanied by a significant decline in proteasomal proteolysis in primary human T lymphocytes, we evaluated the contributory role of USP14 in this decline. Our studies for the first time demonstrate that enzymatic activity of proteasome-associated USP14 is significantly higher in T cells obtained from elderly donors. Additionally, such an increase in USP14 activity could be mimicked by chemically inhibiting the proteasome, using Lactacystin. Thus, USP14 activity appears to be reciprocally regulated by the catalytic function of the 26S proteasome. To determine whether the inhibition of USP14 activity counter regulates proteasomal proteolysis, T cells pretreated with a small molecule inhibitor of USP14, IU1, were activated and assessed for I κ B α degradation as a measure of proteasomal proteolysis. While T cells obtained from young donors demonstrated increased degradation of I κ B α , those from the elderly remained unaffected by IU1 pretreatment. Taken together, these results demonstrate that the decrease in proteolysis of proteasomal substrates during aging is independent of the increased USP14 activity and that the reciprocal regulation of USP14 and proteasomal catalytic activity may be necessary to maintain cellular ubiquitin homeostasis.

Keywords

Aging; T Lymphocytes; Proteasome; Deubiquitinating enzyme; USP14; Ubiquitin

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* **Corresponding author:** Dr. Usha Ponnappan, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 West Markham St., BioMed-I, Slot 511. Little Rock, AR. 72205, USA Phone: 501-296-1252; Fax: 501-686-5359; uponnappan@uams.edu. sponnappan@uams.edu; mpalmieri@uams.edu; dhhsullivan@uams.edu.

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Author contributions

UP designed the research and wrote the manuscript. SP designed the research, performed most of the experiments, analyzed the data and wrote the manuscript. MP coordinated human subjects' recruitment, isolated T lymphocytes and performed some western blot analysis. DHS served as the study geriatrician and assisted in screening and approval of human subjects' enrolled in the study.

1. Introduction

The proteasomal machinery is intricately involved in cellular degradation and also participates in cellular regulation ranging from antigen processing to cell cycle progression (Bedford et al., 2010). For the most part, proteins conjugated to the ubiquitin tag consisting of a minimum of 4 ubiquitin moieties, serve as targets for the 26S proteasome catalytic core (Finley, 2009). Studies have demonstrated that substrates targeted to the proteasome dock at the 19S regulatory subunit by virtue of the presence of ubiquitin receptors, which facilitate in the translocation of the substrate into the internal catalytic chamber for hydrolysis (Glickman et al., 1998; Guterman and Glickman, 2004; Peth et al., 2009). The presence of deubiquitinating activities and ATPases, in association with the proteasome, ensures the unfolding of the protein and enables its translocation, along with the preservation of ubiquitin derived from the tag (Besche et al., 2009; Elsasser et al., 2004; Hanna et al., 2006; Husnjak et al., 2008; Koulich et al., 2008; Lee et al., 2011; Verma et al., 2002). Additionally, proteasome associated deubiquitinating enzymes (DUBs) also act as a timer in permitting the substrates to escape from degradation (Hanna et al., 2006). Substrate deubiquitination, that occurs on the proteasome, has been shown to be mediated by three distinct DUBs i.e., RPN11, UCH37 and USP14, associated with the regulatory particle (Lee et al., 2011). RPN11/POH1 has been demonstrated to cleave at the base of the ubiquitin chain, removing ubiquitin en masse, while UCH37 and USP14 mediate a stepwise removal of ubiquitin from the substrate starting from the distal end. As opposed to UCH37 and USP14 that participate in antagonizing degradation, RPN11 has been demonstrated to promote degradation and shows degradation coupled deubiquitinating activity (Crosas et al., 2006). Evidence suggests that USP14 and UCH37 can both suppress substrate degradation by virtue of removing the ubiquitin tag from targeted proteins, as well as by modulating the functional activity of the proteasome through interference (Hanna et al., 2006). In fact, recent studies employing small molecule chemical inhibitors of USP14 have demonstrated enhanced proteasomal proteolysis, suggesting cross-regulation of the proteasome and USP14 (Lee et al., 2010). Given the central role of proteasome dysfunction during aging in the immune system, we evaluated the contribution of USP14 to the age-associated decline in proteasomal proteolysis observed in primary human T cells. Our studies demonstrate reciprocal regulation of USP14 and functional activity of the proteasome. Employing a small molecule inhibitor of USP14, we now demonstrate enhanced proteasomal degradation of ubiquitinated substrates in T cells derived from young donors but not in those from the elderly. Thus, altered regulation of proteasome-associated USP14 accompanies aging in primary human T lymphocytes and may serve as a compensatory mechanism for regulating free ubiquitin levels in the cell during stress induced by dysfunctional proteasomal proteolysis.

2. Material and Methods

2.1. Antibodies and reagents

Antibody to human USP14 was purchased from Abgent (San Diego, CA). Antibodies to I κ B α , β -actin and Hemagglutinin (HA)-tag were from Santa Cruz biotechnology (Santa Cruz, CA). Antibody to Dansyl-moiety was from Life Technologies (Grand Island, NY). Antibodies to ubiquitin-protein conjugates, 19S and 20S proteasome core subunits were from Enzo Life Sciences (Farmingdale, NY). Protein A/G beads and horseradish peroxidase conjugated anti-rabbit / anti-mouse secondary antibodies were from Thermo Scientific (Rockford, IL). Human TNF α was from ProSpec-Tany Technogene Ltd., (East Brunswick, NJ). IU1, a small molecule inhibitor of USP14, was from BioVision (Mountain View, CA). Lactacystin, a highly specific proteasome inhibitor, was purchased from EMD Millipore (Billerica, MA). Kits for the isolation of CD4⁺ T cells and Naïve and Memory T cell subsets were obtained from Stem Cell Technology (Vancouver, Canada). Electrophoresis supplies

were from Bio-Rad (Hercules, CA). All fine chemicals, unless otherwise mentioned, were obtained from Sigma-Aldrich (St. Louis, MO). Active site probes for assaying deubiquitinating enzyme activity (HAUbVME) and proteasome catalytic activity (Dansyl-Ahx₃L₃VS) were kindly provided by Dr. Hidde Ploegh (Harvard University, USA) and Dr. Huib Ovaal (The Netherlands Cancer Institute, The Netherlands).

2.2. Human subjects

Peripheral blood was obtained by veni-puncture from healthy individuals enrolled from the greater Little Rock area. The age of young donors ranged from 21 to 30 years, and that of the elderly donors ranged from 65 to 89 years. Our study cohorts consisted of 35 young and 35 elderly donors. In consultation with the study geriatrician individuals with asthma and those taking immune-modulating drugs, and other immuno-compromised subjects, were excluded. Subjects taking antibiotics or those who self reported symptoms of recent infection (<3 weeks before enrollment) were also excluded. All protocols involving human subjects were approved by the UAMS Institutional Review Board and appropriate informed consents were obtained. Blood was drawn at the Clinical Research Center (CRC) at UAMS.

2.3. T lymphocyte isolation

CD4⁺ T cells were isolated by negative selection from blood using the EasySep CD4⁺ T cell enrichment kit according to the manufacturer's recommended protocol. Isolated CD4⁺ T cells were consistently 90-98% pure. CD4⁺CD45RO⁺ and CD4⁺CD45RA⁺ T cells were also isolated by negative selection from blood using EasySep Human Memory CD4⁺ T cell enrichment kit and EasySep Human Naïve CD4⁺ T cell enrichment kit, respectively, according to the manufacturer's recommended protocol. Isolated CD4⁺CD45RO⁺ / RA⁺ T cells were 90-95% pure. Typically, isolated T cells represented 80-85% of PBMC, with an average of 85% being CD4⁺ T cells, in the young donor population. In the elderly group, T cells represented 80-83% of PBMC with 84% of those being CD4⁺ T cells. Of the CD4⁺ T lymphocytes, CD45RA⁺, CCR7⁺, CD45RO⁻ subset and CD45RO⁺, CD45RA⁻ subset represented an average of 52-54% and 46-48%, respectively, in the young and 48-50% and 50-52% respectively, in the elderly donor group.

2.4. Preparation of cytosolic lysates and Western Blotting

Isolated T cells / T cell subsets were washed in ice-cold phosphate buffered saline and homogenized in lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1mM sodium orthovanadate, 0.5% NP-40, 0.5 mM DTT, 5mM NEM and protease inhibitor cocktail. Nuclei and cellular debris were removed by centrifugation and the protein concentration was determined using BioRad protein assay. T cell lysates, equalized for protein levels (25µg), were resolved using Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, immunoblotted with specific antibody and detected using an appropriate secondary antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence.

For assaying proteasome-associated proteins, CD4⁺ T cells were washed in ice-cold phosphate-buffered saline and homogenized in proteasome lysis buffer containing 50mM Tris-Cl (pH 7.4), 5mM MgCl₂, 250mM Sucrose and 2mM ATP. T cell proteasome lysates (150µg protein) were subjected to immunoprecipitation using an antibody to 20S proteasome core subunits. The immunoprecipitate complex was captured using protein A/G beads and subjected to western blotting as detailed above.

2.5. Labeling of cells with the proteasome activity profiling probe for assessing proteasome catalytic activity

Catalytic activity of proteasomes was assessed in living cells using a cell-permeable proteasome activity profiling probe bearing a dansyl-tag as described (Ponnappan et al., 2007). Briefly, T cell subsets (20×10^6 cells) were incubated in conditioned RPMI 1640 medium containing 10% fetal bovine serum, which was supplemented with $50\mu\text{M}$ of the activity profiling probe, DansylAhx₃L₃VS, for 2h at 37°C. Labeled cells were washed twice with cold phosphate-buffered saline, pelleted by centrifugation, and lysed in a buffer containing 10mM Tris-HCl (pH 7.8), 140mM NaCl, 0.5% NP-40 and protease inhibitors. Protein concentration of the lysates was determined using BioRad protein assay. Proteasome activity was assayed by immunoblotting the lysates (Berkers et al., 2005). Briefly, lysates equalized for protein (25 μg) were resolved on 12% gels using SDS-PAGE, immunoblotted using an antibody to the dansyl moiety and detected using enhanced chemiluminescence.

2.6. Assaying deubiquitinating enzyme activity associated with the proteasome employing activity profiling probe in T cells

Deubiquitinating enzyme/s activity was assessed in T cells by employing an active site specific probe (HAUbVME) developed by Dr. Ploegh's group (Borodovsky et al., 2001). Isolated T cells were washed with phosphate buffered saline and homogenized in lysis buffer containing 50mM Tris-Cl (pH 7.4), 5mM MgCl₂, 250mM Sucrose, 2mM ATP and 1mM DTT. Nuclei and cellular debris were removed by centrifugation. Lysates (25 μg protein) were labeled with 2 μl of a 0.2mg/ml stock of the activity profiling probe (HAUbVME) at 37°C for 1h. One set of samples pretreated with 500nM of ubiquitin aldehyde, a specific inhibitor of DUBS, for 30 min at room temperature prior to labeling with the probe, served as a specificity control. HAUbVME-labeled lysates were then subjected to immunoprecipitation using an antibody to 20S proteasome core subunits by incubation with the antibody overnight at 4°C, followed by capture using ProteinA/G beads. The immunoprecipitate complex was resuspended in Laemmli SDS-sample buffer, heated for 5 min in a boiling water bath and subjected to SDS-PAGE and immunoblotted using antibody to HA.

2.7. Statistical analyses

Differences between means of the data generated in the study were analyzed using Student's *t*-test. Differences were considered significant, if $p < 0.05$.

3. Results

3.1. Proteasome associated catalytic activity declines with age in both memory and naïve CD4⁺ T cell subsets

As our previous studies using enriched proteasome preparations have demonstrated that aging is associated with a significant decrease in proteasomal chymotryptic activity in unfractionated T cells (Ponnappan et al., 1999), we employed a novel cell permeable active site probe for the analyses of proteasomal catalytic activity in naïve and memory human T cell subsets obtained from young and elderly donors. Use of such probes would obviate the variability introduced by cell manipulations employed in proteasome enrichment, providing us with a more accurate measure of proteasomal proteolytic activity. As seen in Fig. 1A, all active catalytic subunits of the proteasome are labeled in T cell subsets obtained from both young and elderly donors, however, the levels of probe binding, which is a direct measure of catalytic activity, are significantly lower in cells obtained from the elderly relative to those from young donors (Fig. 1B). Interestingly, both CD45RA⁺ and CD45RO⁺ T cell subsets from the elderly, demonstrated lower activity and is in keeping with our previously reported

values from enriched proteasomal preparations (Ponnappan et al., 1999). In contrast, protein levels of C2 (α -6 subunit of the 20S proteasome) present in cells appeared to be unaffected by the age of the donor.

3.2. Cellular levels of ubiquitinated proteins are higher in T cells and T cell subsets from the elderly, when compared to those from the young

As lowered functional activity of the proteasome impacts polyubiquitinated protein removal in the cells, we next analyzed overall cellular ubiquitinated protein levels in T cells and T cell subsets obtained from young and elderly donors. As a minimum of 4 ubiquitin moieties have been demonstrated to be required for proteasomal targeting and degradation (Thrower et al., 2000), we evaluated ubiquitinated proteins that exhibit 4 or more ubiquitin tags (Molecular weight >28kDa). Polyubiquitinated protein levels were evaluated in primary CD4⁺ and CD8⁺ T cells as well as in CD4⁺ CD45RA⁺ and RO⁺ T cell subsets. As shown in Fig. 2A and B, ubiquitinated protein accumulation was significantly higher in CD4⁺ and CD8⁺ T cells obtained from the elderly, when compared to those from young donors. Similarly, as shown in Fig. 2C and D, ubiquitinated protein accumulation was also higher in both naïve and memory T cell subsets from the elderly relative to those from the young. While CD4⁺CD45RO⁺ T cell subset from the elderly demonstrate highest accumulation of overall ubiquitinated proteins, CD4⁺CD45RA⁺ T cell subset also demonstrate significantly higher levels when compared to similar cells from young donors. Thus, overall levels of ubiquitinated proteins appear to be higher in T cells and T cell subsets from the elderly, and this accumulation could be attributed to decreased proteasomal proteolysis in these cells. Furthermore, since deubiquitinating enzymes associated with the proteasome are responsible for ubiquitin trimming from substrates targeted to the proteasome for degradation, and in light of growing evidence that the manner in which proteasome-associated deubiquitinating enzymes, USP14 and UCH37, deubiquitinate substrates can in fact suppress/delay degradation and modulate proteasome function, we decided to next analyze the functional activity of the proteasome-associated deubiquitinating enzyme USP14.

3.3. Proteasome associated deubiquitinating enzyme USP14 demonstrates significant increase in activity during aging

The functional activity of proteasome-associated deubiquitinating enzyme USP14 was assessed in CD4⁺ T cells employing an active site probe as detailed in the experimental methods section. Based on the levels of probe binding to the active sites of the enzyme, which is a direct measure of the enzymatic activity, and as shown in Fig. 3A and B, functional activity of proteasome-associated USP14 was significantly higher in cells obtained from elderly donors relative to those from the young. In analyzing if the observed increase in the functional activity of proteasome-associated USP14 was either due to higher protein levels of USP14 associated with the proteasome or due to an overall increase in cellular levels of USP14, we found that neither was affected by the age of the donor (Fig. 3C and D). To determine whether cellular levels of USP14 are differentially regulated during aging, we labeled cell lysates with the active-site specific probe and assessed USP14 levels using an antibody. Data presented in Fig. 3E demonstrates that cellular USP14 exists both as active (bound to the probe - 64kDa) and inactive (not bound to the probe - 56 kDa) forms and can be detected by virtue of their differential electrophoretic mobility. The levels of inactive form of USP14 were lower in T cells from young donors relative to those from the elderly.

To further test whether the observed increase in proteasome associated USP14 activity could be attributed to lowered proteasomal proteolytic function during aging, we employed CD4⁺ T cells obtained from young donors. These cells were incubated with a cell permeable 26S proteasome inhibitor, lactacystin, for 2h prior to labeling with the active site probe for assaying DUB activity. As shown in Fig. 3F and G, USP14 activity significantly increases in

the immunoprecipitates of proteasomes following inhibition, as demonstrated by increased binding of probe to the enzyme. This observation in primary human T cells confirms data previously reported in Jurkat T cell line (Borodovsky et al., 2001).

Taken together, these results demonstrate increased proteasome-associated USP14 activity in CD4⁺ T cells from the elderly, however, the levels of USP14 found in association with the proteasome or total cellular USP14, appeared unaffected by the age of the donor. Increased USP14 activity associated with the proteasome during aging in T cells may be attributed to lowered proteasomal proteolytic activity.

3.4. Increased USP14 functional activity observed in T cell subsets from the elderly may contribute to altered levels of free cellular mono-ubiquitin during aging

As USP14/Ubp6 has been shown to be activated 300-fold upon binding to the proteasome, participating in the stepwise removal of ubiquitin and sparing it from proteasomal degradation (Hu et al., 2005), and that a loss of USP14 activity results in reduced levels of free ubiquitin (Anderson et al., 2005; Hanna et al., 2007), we next analyzed free ubiquitin levels in CD4⁺ T cells during aging, to evaluate whether increased USP14 activity associated with the 26S proteasome, impacts levels of free cellular ubiquitin. As shown in Fig. 4, free ubiquitin was observed in cells obtained from both young and elderly donors; however, the levels of free ubiquitin observed in cells from the elderly were far higher than those from the young, irrespective of the T cell subset. This observation is in line with the increased functional activity of proteasome-associated USP14 in CD4⁺ T cells from the elderly, resulting in regeneration of ubiquitin from targeted substrates.

3.5. Small molecule inhibitor of USP14, IU1, enhances proteasomal proteolysis of ubiquitinated substrate in T cells obtained from young but not those from elderly donors

As increased free cellular ubiquitin levels have been attributed to increased DUBs activity, specifically those of USP14 found in association with the proteasome, we next evaluated the contribution of USP14 in proteasome-mediated degradation of a classical substrate, polyubiquitinated I κ B α . CD4⁺ T cells obtained from young and elderly donors were pretreated with IU1, a small molecule inhibitor of USP14. Following treatment with the inhibitor, T cells were activated with either TNF- α for 15 min or Phorbol myristate acetate + Ionomycin for 20 min., both potent activators of the NF κ B signaling pathway. Cell lysates obtained following activation were resolved using SDS-PAGE, and immunoblotted with an antibody to I κ B α . As shown in Fig. 5A - D, CD4⁺ T cells from young donors, but not those from the elderly, demonstrated significant disappearance of the I κ B α protein, indicative of enhanced proteasomal degradation following pretreatment with IU1, relative to those in cells without pretreatment, under both activation protocols. While degradation of I κ B α , induced by TNF α and PMA+Ionomycin, was higher in T cells from young donors, pretreatment with IU1 in the absence of activation, did not affect the levels of I κ B α in cells from young or elderly donors. These observations demonstrate that inhibition of the functional activity of USP14 by IU1 delays the trimming of ubiquitinated substrates, thus retaining the targeted substrates for a longer duration on the regulatory cap of the proteasome, i.e. enhances the dwell time for targeted substrates on the proteasome. When proteasomes are fully functional, as observed in T cells from young donors, such retention or increase in dwell time, allows for increased overall substrate degradation. However, in cells obtained from the elderly, despite the increase in time of retention of the substrate on the 26S proteasome, enhanced degradation of the targeted substrates is not observed, due to intrinsic defects in proteasomal catalytic activities.

4. Discussion

In this study we have addressed the regulatory role of USP14, a proteasome associated deubiquitinating enzyme, during aging. Aging is accompanied by a significant decline in proteasomal proteolysis in a variety of cell types including lymphocytes, which has often been attributed to chronic oxidative stress (Ponnappan and Ponnappan, 2011; Stone et al., 2004). Studies on proteasome associated proteins, such as USP14, have been demonstrated to play important roles, both catalytically and non-catalytically, in modulating proteolytic function of the 26S proteasome (Borodovsky et al., 2001; Hanna et al., 2006). As most of the previous reports on USP14 have been largely restricted to findings in yeast and tumor cell lines, we now provide information on the regulatory role of this Ubiquitin specific protease in primary human T cells. Studies reported in this manuscript were designed to explore the potential contribution of USP14 to the regulation of proteasome function during aging.

Previous studies employing tumor cell lines, have demonstrated the participation of USP14 in deubiquitinating proteasomal substrates, and that Ubp6, the USP14 ortholog in yeast, interferes with proteasomal proteolysis by delaying the degradation of targeted substrates (Hanna et al., 2006). Additionally, these studies demonstrated that the levels of Ubp6 are modulated by free ubiquitin via a feedback regulatory circuit (Hanna et al., 2003; Hanna et al., 2007). Employing primary human CD4⁺ T cells, for the first time, we have now uncovered a complex reciprocal regulation of USP14 function and proteasomal enzymatic activity during aging. We also demonstrate an age-related functional modulation of proteasome associated USP14 activity, which directly impacts free ubiquitin levels in T cells from the elderly. Interestingly, proteasome associated USP14 activity was significantly higher in CD4⁺ T cells from the elderly, relative to those from the young. Increased USP14 functional activity has previously been demonstrated to regulate the proteasomes by directly down regulating proteolysis, or indirectly, by regulating deubiquitination of substrates targeted to the proteasome (Hanna et al., 2006). To decipher USP14-mediated regulation of proteasomal degradation in primary CD4⁺ T cells from the elderly, we employed a small molecule inhibitor of USP14, IU1. These studies further confirmed two observations noted independently, i.e., (1) that inhibition of USP14 up-regulates proteasomal proteolysis in the cells from young donors, perhaps by virtue of 20S gate opening, but fail to do so in cells from the elderly, and (2) that inhibition of USP14 in T cells from both young and elderly donors influences the levels of poly-ubiquitinated proteins and free ubiquitin. Taken together, these results imply that increased functional activity of proteasome-associated USP14 observed in the elderly, is not the sole reason for lowered proteasomal proteolysis, and that defects intrinsic to the 26S proteasome complex may play a significant role. As USP14 mediated up-regulation of proteasomal enzymatic function, by specific inhibitors of USP14, has been proposed as a potential mechanism for reversing proteasomal dysfunction under conditions such as Alzheimer's disease and other protein accumulation pathologies (Lee et al., 2010), failure to effectively enhance proteasomal proteolysis in cells from the elderly, should serve as a harbinger that defects intrinsic to the proteasome, in addition to proteasomal - interacting proteins, may play crucial roles in the defects that accompany aging and under pathological conditions such as Alzheimer's disease. Future studies should address whether agents that up-regulate proteasomal proteolysis by the induction of proteasomal subunits, can be used in conjunction with USP14 inhibitors to effectively enhance proteasomal catalysis. With the identification of a complex regulatory mechanism between the 26S proteasome and USP14, it begs the question of how other proteasome interacting DUBs, such as UCH37, that has been implicated in deubiquitinating substrates targeted to the proteasome, is impacted by aging (Hamazaki et al., 2006; Stone et al., 2004). Future studies should attempt to define the contribution of this DUB to proteolysis mediated

by the 26S proteasome and provide information about the evolutionary importance of having more than one DUB at the 19S regulatory cap of the proteasome.

In conclusion, these findings on the regulatory role of USP14 provide direct evidence that deubiquitination and proteolysis, within the 26S proteasome, are tightly linked. Thus, age-associated alteration in the catalytic core impacts USP14 association and functional activity, aiding in the trimming of ubiquitin chains to help restore cellular ubiquitin levels.

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Abbreviations

DUB	Deubiquitinating enzyme
USP14	Ubiquitin Specific Protease 14
UCH	Ubiquitin Carboxyl-terminal Hydrolase
RPN	Regulatory Particle Non-ATPase

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Highlights

- ▶ Proteasome-associated USP14 activity increases in human T cells during aging.
- ▶ Enzymatic activities of USP14 and 26S proteasome are reciprocally regulated.
- ▶ Inhibiting USP14 activity does not ameliorate proteasome dysfunction during aging.
- ▶ Observed increase in USP14 activity with age is required for ubiquitin homeostasis.

Fig. 1. Catalytic activity of the proteasome decreases with age in both naïve and memory human CD4⁺ T cell subsets

A. Catalytic activity of the proteasome was assessed in live T cell subsets using a cell permeable active site-directed probe, Dansyl-AhX₃L₃-VS, followed by immunoblotting using an anti-dansyl antibody. Each lane represents 25μg of total cellular protein. Western blotting of C2 subunit of the proteasome is provided to demonstrate equal amounts of proteasome subunits were present in the lysates. β-actin was used as a loading control. Constitutive and inducible catalytic subunits β2, β2i, β5, β5i and β1, β1i are depicted from one representative young and old donor pair. **B.** Densitometry data (mean ± SE) and statistical analyses of proteasome catalytic activity derived from a minimum of 5

independent donor pairs are plotted. * denotes statistical significance between the young and elderly group at $p < 0.05$.

Fig. 2. Poly-ubiquitinated protein profile in T cell subsets obtained from young and elderly donors

T cells obtained from young and elderly donors were homogenized and total cell lysates were prepared. Lysates (25μg protein) were immunoblotted with an antibody to ubiquitin following SDS-PAGE. **A.** Representative poly-ubiquitinated protein profile of CD4⁺ and CD8⁺ T cell subsets obtained from one young and elderly donor pair is presented. β-actin is provided as a loading control. **B.** Integrated densitometry and statistical analyses of the data (mean ± SE) from a minimum of five independent donor pairs are provided. ** denotes statistical significance between the young and elderly group at p<0.002. **C.** Representative poly-ubiquitinated protein profile of CD4⁺ CD45RA⁺ and RO⁺ T cell subsets from one

young and elderly donor pair is presented. β -actin is provided as a loading control. **D.** Integrated densitometry and statistical analyses of the data (mean $\pm$ SE) from a minimum of five independent donor pairs are provided. ** denotes statistical significance between the young and elderly group at $p < 0.001$.

Fig. 3. Activity of proteasome-associated deubiquitinating enzyme, USP14, in human CD4⁺ T cells obtained from young and elderly donors

CD4⁺ T cell lysates obtained from young and elderly donors, equalized for protein levels, were labeled with the deubiquitinating enzyme active site-directed probe, HAUbVME. Labeled lysates were subjected to immunoprecipitation using an antibody to 20S proteasome core. Proteasome immunoprecipitates containing labeled proteasome-associated deubiquitinating enzymes were resolved using SDS-PAGE and immunoblotted using an anti-HA antibody. Identity of the protein was derived from previously published data and western blotting using USP14 antibody.

A. Representative blot from one immunoprecipitation analysis, derived from a pool of a minimum of 3 donors, is provided.

B. Integrated densitometry and statistical analyses of data (mean $\pm$ SE) from three independently performed immunoprecipitations and immunoblots are provided. ** denotes statistical significance between the young and elderly group at $p < 0.001$.

C & D. Protein levels of USP14 in immunoprecipitated proteasomes (C) and crude cellular lysates (D) of CD4⁺ T cells obtained from young and elderly human donors. Levels of USP14 protein in total cell lysates, as well as in immunoprecipitated proteasome preparations were detected by immunoblotting using an antibody specific to USP14, following SDS-PAGE. Representative data from one young and elderly donor pair are provided. Blots were stripped and reprobed with antibody to β -actin (cellular lysates) or PA28 α (proteasome immunoprecipitates) to demonstrate equal protein loading. Blots (proteasome immunoprecipitates) reprobed with antibody to TBP7 indicate 19S subunit expression. A total of four independent immunoprecipitation analyses were performed for proteasome-associated USP14, and a minimum of 7 pairs of young and elderly donors were employed for the western blot analyses of cellular USP14.

E. Western blot of USP14 in cell lysates from young and elderly donors following labeling with HAUBVME was carried out to demonstrate active and inactive forms of USP14 and are indicated by arrows.

F. Active site labeling of proteasome-associated USP14 is increased upon proteasome inhibition in T cells from young donors. CD4⁺ T cells from young donors were pretreated with lactacystin (5 μ M; 2h) to inhibit the proteasome. Total cell lysates were prepared followed by active site labeling of DUBs using the HAUBVME probe. Proteasomes were immunoprecipitated as described, and subjected to immunoblotting using anti-HA antibody, following SDS-PAGE. Representative western blot obtained from one young donor before and following treatment with lactacystin, out of 4 independent samples analyzed, is provided.

G. Integrated densitometry and statistical analyses of data (mean $\pm$ SE) from four independently performed immunoprecipitations and immunoblots are provided. * denotes statistical significance at $p < 0.02$.

Fig. 4. Effect of age on the levels of free ubiquitin in CD4⁺ T cell subsets

Total cell lysates were prepared from CD4⁺CD45⁺ RA⁺ and RO⁺ T cell subsets obtained from young and elderly donors. Lysates (25μg protein) were resolved using SDS-PAGE and immunoblotted using an anti-ubiquitin antibody. Representative blot from one young and elderly donor pair out of 5 pairs tested is provided. Arrow indicates free ubiquitin (Ub¹). β-actin is provided as a loading control

Fig. 5. Effect of small molecule inhibitor of USP14, IU1, on activation-induced IκB-α degradation during aging

CD4⁺ T cells obtained from young and elderly donors were either pretreated with IU1 (50μM) for 5h or left untreated. At the end of incubation, cells were subjected to an activation protocol using either PMA+ ionomycin (20 min activation) or TNF-α (15 min activation) or left unactivated. At the end of the activation protocol, cells were washed and lysed as described. Lysates (25μg protein) were subjected to SDS-PAGE. Resolved proteins were immunoblotted using an antibody to IκB-α. Representative western blots obtained following various treatment combinations / activation protocols, of one donor pair out of a minimum of 5 pairs tested, are provided. β-actin levels serve as loading controls. (A & B: PMA + Ionomycin treatment; C & D: TNF-α treatment). Panels B and D depict percent degradation of IκB- α (mean ± SE) relative to untreated controls representing 0% degradation. * denotes statistical significance at p<0.02. ** denotes statistical significance at p<0.001. NS: Not Significant.