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Redox regulation of the proteasome in T lymphocytes during aging

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Abstract

Proteasome is a major cellular organelle responsible for the regulated turn-over of both normal and misfolded proteins. Recent reports from our laboratory have implicated lowered proteasomal chymotryptic activity to be responsible for decreased induction of the transcription factor NFκB in T lymphocytes during aging. In this study, we have further analyzed the basis for this decline in proteasomal function, by focusing on the role of oxidative stress. Upon exposure to the pro-oxidant BSO, both ATP-stimulatable 26S, as well as ATP-independent 20S proteasomal catalytic activity could be down-regulated in T cells from young donors, mimicking the decline observed in T cells from the elderly. Loss in these catalytic activities, following exposure to pro-oxidant stimulus also resulted in a decline in both activation-induced proliferation as well as degradation of the inhibitor IκBα, with concomitant increase in the accumulation of carbonylated proteins, mimicking responses seen in T cells from the elderly. Pretreatment with an antioxidant, NAC could override pro-oxidant-mediated, but not age-associated decrease in both 20S proteasomal activities. These results suggest that the decrease in proteasomal activities observed during aging may be secondary to oxidative stress and underlie immune senescence.

Keywords

Aging; proteasome; oxidative stress; GSH; ROS; T lymphocytes; immune-dysregulation; immune senescence

Introduction

Aging is characterized by a ubiquitous decline in the functional capacity of various physiological compartments including the immune system. Previous studies have clearly demonstrated that aging is accompanied by deficits in activation-induced T cell responses such as of IL-2R expression, IL-2 secretion and proliferation [1–8]. We have previously demonstrated that decreased induction of the transcription factor NFκB, contributes to lowered induction of IL-2R during aging [4,9]. Additional studies demonstrated this decline in NFκB to be attributable to a central defect in the proteolytic activity of the 26S proteasome [4,7,10].

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To further delineate the basis for the decline in proteasomal proteolysis, we have now focused our efforts on the role of oxidative stress and the impact of redox regulation on the 26S proteasome function during aging. The 26S proteasome, a multi-subunit protease complex, consists of a 20S catalytic core flanked by 19S regulatory subunits. The 20S core is a barrel-like particle consisting of a stack of four rings made up of two outer α -rings and two inner β -rings. The two inner β -rings contain three pairs of active sites (β_1 , β_2 , and β_5) that perform distinct proteolytic activities namely peptidyl glutamyl peptide hydrolyzing (or caspase-like), trypsin-like and chymotrypsin-like activity, for cleavage after acidic, basic, and hydrophobic amino acids, respectively [11–13].

Besides targeted degradation of regulatory proteins, an important function of the proteasome is the degradation of oxidized and aberrant proteins [14–17]. Increased accumulation of highly oxidized and cross-linked protein aggregates within the cell observed during aging has been attributed to decreased proteasome function [18,19]. Indeed, both 20S and 26S proteasomes show age-related decline in their enzymatic activities in most cell types ranging from T lymphocytes to neurons [4,10,20–22]. Studies by Chondrogianni *et al* revealed that, while the inhibition of proteasome induced a senescent phenotype, stable expression of β_1 or β_5 catalytic subunits in WI38 immortalized fibroblasts and HL60 leukemic cell line results in increased proteasomal activities and cell survival following treatment with oxidants [23]. Additionally, a recent study by the same group demonstrated that over-expression of β_5 subunit in primary IMR90 human fibroblasts delays replicative senescence by 4–5 population doublings [24]. Taken together, these studies underscore the importance of proteasome in the progression of the senescent phenotype by regulating cell survival and protection from oxidative stress.

While several studies have now documented declines in proteolytic function of the proteasome during advancing age, few studies, if any, have focused on the underlying basis for this decline. Oxidative stress has often been implicated in the functional loss accompanying aging, more specifically the involvement of thiols in T cell responsiveness to external stimuli [25–29]. However, there is little evidence for the effect of oxidative stress on proteasomal activities in primary human T lymphocytes. The free radical theory of aging postulates that the ubiquitous and progressive decline in the functional capacity of cells during aging is a consequence of oxidative damage caused by ROS [30]. Thus, the proteasome by virtue of being the organelle responsible for the removal of oxidatively modified proteins may itself be a direct target of oxidative stress during aging. We therefore investigated the effect of oxidative stress on proteasomal catalytic activity in T lymphocytes from young and elderly donors.

We report here that upon exposure to pro-oxidant conditions, T cells from young donors mimic responses seen under basal conditions in T cells from elderly. These include a decrease in GSH content, increased accumulation of carbonylated proteins, increased generation of ROS and a decline in proteasomal catalytic activities. Furthermore, pretreatment with N-acetyl cysteine (NAC), afforded protection against pro-oxidant-induced, but not age-associated decrease in proteasomal activities. The difference in the effect of NAC may reveal alterations induced by acute, as opposed to chronic oxidative stress occurring throughout the life of an individual. Thus, modulation of proteasome function by altered redox status may contribute to immune dysfunction in senescence.

Materials and Methods

Reagents

Antibodies to CD19 and human IgM were obtained from Southern Biotech Associates (Birmingham, AL). Anti-CD3 was obtained from Zymed (San Francisco, CA); anti-CD28 and anti-I κ B α were obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-di-nitro-phenyl (DNP) was obtained from Molecular Probes (Eugene, OR) and anti-IgG coupled to horseradish

peroxidase was obtained from BD-Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence (ECL) reagents were from Amersham (Arlington Heights, IL). Luciferase reporter assay kit was purchased from Promega (Madison, WI). 2, 7-dichlorodihydrofluorescein diacetate (DCHF-DA) was obtained from Molecular Probes (Eugene, OR). TNF- α was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Proteasome IP kit was from EMD Biosciences Inc. (San Diego, Ca). Ada-lys-(Biotnyl)-(Ahx)₃-(Leu)₃-Vinyl sulfone, was from Biomol, (Plymouth Meeting, PA).

All fine chemicals, unless otherwise mentioned, were obtained from Sigma Chemical Co. (St Louis, MO). Succinyl-leu-leu-val-tyr-4-amido-methyl-coumarin (Suc-LLVY-AMC) was from Bachem Biosciences (King of Prussia, PA). Electrophoresis supplies were from BioRad (Richmond, CA). ILU-18 cell line was obtained from Dr. Charles O' Brien (UAMS) [31].

Human subjects

Peripheral blood was obtained by venipuncture from healthy individuals. Young donors were 21–30 years of age and elderly donors were 65–80 years of age. Our donor population consisted of an equal representation of males and females. Both young and elderly donors were in good physical and mental health, had no apparent illness as suggested by an elaborate screening history and were on no medication directly impacting the immune function during the course of this study. A minimum of five donor pairs were used in each experiment. The protocol for blood draw from human subjects was approved by the IRB (UAMS) and blood was drawn at the local General Clinical Research Clinic (GCRC).

T lymphocyte isolation

Peripheral blood mononuclear cells (PBMC) were obtained and T lymphocytes were enriched as previously described [32]. Briefly, PBMCs obtained from young and elderly donors were subjected to panning using anti-human IgM on plastic petri dishes to obtain partially purified T lymphocytes. These cells were then subject to a second round of negative selection using anti-CD19 magnetic beads. Isolated T cells were found to be 90–95% CD3 positive as determined by flow cytometry using FITC-labeled CD3 antibody.

Measurement of T lymphocyte proliferation

T lymphocytes (1×10^5 /100 μ l/well) were stimulated with immobilized antibody to CD3 and CD28 (1 μ g/ml) for 48 h at 37°C in a 96-well plate. Radio-labeled ³H-thymidine (1 μ Ci/well) was added to the cells during the last 6 h of culture, and incorporated radioactivity was measured using a dry-matrix beta counter (Packard, Pangbourne, UK). Proliferation index was calculated as a ratio of counts obtained in anti-(CD3+ CD28) activated cultures to those in untreated control cultures.

Measurement of intracellular GSH

Intracellular GSH levels were assayed as described [33]. Briefly, 100 μ l of ice-cold lysis buffer was added to T cells (1×10^6), incubated on ice for 10 min, and centrifuged at 12,000 $\times$ g for 10 min in a microfuge. To this preparation, 2 μ l of 25 mM monochlorobimane (MCB) and 2 μ l of a 50U/ml glutathione-S-transferase (GST) stock, were added and incubated at 37°C for 30 min. At the end of incubation, fluorescence was measured using a fluorescence plate reader (TECAN, NC.) at an excitation wavelength of 340 nm and emission wavelength of 465 nm. Values obtained in the absence of GST and MCB served as negative controls.

Measurement of intracellular ROS using DCHF-DA

T lymphocytes (1×10^6 /ml) were incubated with 10 μ M DCHF-DA at 37°C for 30 min in dark [34]. At the end of incubation, cells were washed and resuspended in PBS. ROS generation

was monitored following addition of BSO (200 μ M) and detected using a spectrofluorometer (Perkin Elmer Corporation, Norwalk, CT) at an excitation wavelength of 480 nm and emission wavelength of 525 nm. Generation of ROS is expressed as mean fluorescence intensity (MFI) at various time points.

Determination of 20S proteasome catalytic activities

T cells were lysed using proteasome lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , and 250 mM sucrose followed by centrifugation at $12,000\times g$ for 10 min [4]. The supernatant was then subjected to ultra centrifugation at $100,000\times g$ for 1 h at 4°C . 20S proteasome-enriched fraction (50 μ g protein) was incubated in 200 μ l of assay buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl_2 , and 1 mM dithiothreitol (DTT) for 1 h at 37°C in the absence of ATP. Fluorogenic peptide substrate (Suc-LLVY-AMC, Bz-R-AMC or N-LLE- β NA, 40 μ M) was added, and incubated for 30 min at 37°C . The reaction was quenched with 1 ml of ice-cold ethanol. Hydrolysis of the fluorogenic peptide was determined by measuring fluorescence using spectrofluorometer at an excitation wavelength of 380 nm and emission wavelength of 440 nm for AMC substrates, and at an excitation wavelength of 335 nm and emission wavelength of 410 nm for β -NA substrate. A proteasome specific inhibitor was employed to ensure specificity of the assays.

Determination of 26S proteasome activities

26S proteasome-enriched fractions were prepared as described earlier [4] or by immuno-precipitation employing the proteasome isolation kit (EMD Biosciences, CA). Briefly, T cells were lysed using proteasome lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 250 mM sucrose and 2 mM ATP and subjected to centrifugation at $12,000\times g$ for 10 min. The supernatant was subsequently subjected to ultra centrifugation at $100,000\times g$ for 6 h at 4°C . At the end of centrifugation, the pellet fraction containing 26S proteasome was resuspended in buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl_2 , 20% glycerol and 2 mM ATP and subjected to immuno-precipitation using the isolation kit, per manufacturer's protocol. Proteasome enriched preparations were Western blotted, and assayed for catalytic activity in 3 pairs of young and elderly donors, using 5 μ M lactacystin or MG132 to establish specificity (Fig. 2A[suppl.] & 2B[suppl.]). Proteasome specific catalytic activity using fluorogenic substrate was found to be similar in enriched preparations of proteasome obtained either by immuno-precipitation or stepwise ultracentrifugation. 26S enriched fraction or immune complexes (50 μ g protein) were incubated in 200 μ l of assay buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl_2 , 1 mM DTT, and 0.5 mM of the fluorogenic peptide substrate, in the presence or absence of 2 mM ATP (dose determined to be maximal for activity; Fig. 1 [suppl.]). After incubation for 1 h at 37°C the reaction was quenched with 1 ml of ice-cold ethanol. Hydrolysis of fluorogenic peptide substrates and specificity of the assay were determined as described [4]. ATP specific activity was obtained by using apyrase, and proteasome specific activity was calculated following subtraction of activity obtained in the presence of apyrase and was completely inhibited in the presence of proteasome inhibitor MG132 or LLnL.

Proteasome specific activity was also evaluated in Jurkat T cells by employing a novel biotinylated proteasome activity profiling probe [35]. Jurkat T cells (20×10^6), either untreated or treated with 200 μ M BSO for 24h, with or without 5mM NAC pretreatment, were washed twice with cold phosphate-buffered saline, pelleted by centrifugation, and lysed in buffer containing 50mM Tris-HCl (pH 7.4), 5mM MgCl_2 , 250mM sucrose and 2mM ATP. Lysates from Jurkat cells pre-treated with 5 μ M Lactacystin for 2 h and labeled with the biotinylated probe served as a specificity control. Cell lysates equalized for protein (30 μ g) were labeled with 5 μ M biotinylated probe in a reaction buffer containing 50mM Tris-HCl (pH 7.4), 5mM MgCl_2 , 2mM ATP and 2mM DTT for 2h at 37°C . Labeling reactions were quenched by the

addition of SDS-sample buffer and boiling for 5 min. Samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with streptavidin conjugated to horseradish peroxidase (HRP) followed by ECL.

Detection of activation-induced I κ B α degradation by Western blotting

Cytosolic extracts for Western blotting were prepared by homogenization of cells in lysis buffer containing 1 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 0.5% NP-40, 0.5 mM DTT, 0.5 mM phenyl methyl sulphonyl fluoride, and 10 μ g/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor [4]. Protein content of cytosolic extracts was determined using Biorad protein assay. Cell lysates equalized for protein (30 μ g), were resolved by 10 % SDS- PAGE, transferred to nitrocellulose membrane, immunoblotted with anti-I κ B α antibody and detected using anti-IgG coupled to HRP followed by ECL.

Detection of carbonylated protein by Western Blotting

Carbonylated proteins were detected using OxyBlotTM protein oxidation detection kit (Chemicon, Temecula, CA), per manufacturer's protocol. Briefly, T cells were lysed in 6 % SDS and 2, 4-dinitrophenyl-hydrazine (DNPH) was added to derivatize the carbonyl groups in the protein side chains to 2, 4-DNP-hydrazone. DNP-derivatized proteins were resolved by 12 % SDS-PAGE and detected by Western blotting using anti-DNP antibody followed by anti-rabbit-IgG-HRP and ECL.

Measurement of NF κ B- dependent transcriptional activity using luciferase reporter assay

ILU-18 cells were derived from a bone marrow stromal cell line called +/- LDA.11 and transfected with murine IL-6 minigene containing the luciferase promoter and 3'UTR. ILU-18 cells were either left untreated or treated with TNF- α (20 ng/ml) for 24 h at 37°C. Lysates were prepared using reporter lysis buffer and luciferase reporter activity was measured employing 50 μ g protein, per manufacturer's protocol (Promega, Madison, WI).

Statistical analysis

A minimum of 5 samples per age group was analyzed for each facet of the study. Statistical analysis was performed using ANOVA (one-way or two- way, wherever appropriate), followed by Student Newman-Kuel test for multiple pair-wise comparisons. Probability of $p < 0.05$ was considered to be significant.

Results

1. Pro-oxidant treatment decreases intracellular levels of GSH and up regulates ROS generation in T lymphocytes

As levels of GSH and induction of ROS are counter-regulated in the cell, we first examined the basal levels of GSH in T cells from young and elderly donors. Our results demonstrate that constitutive levels of GSH in T cells from the elderly were significantly lower than those in young donors, consistent with previous reports in murine T cells [36]. Upon exposure to a mild pro-oxidant, BSO for 24 h, T lymphocytes obtained from young donors exhibited a significant decrease in the levels of GSH at 24 h, when compared to untreated cells (Fig 1a). T cells from elderly donors exposed to BSO failed to further down regulate GSH levels. In contrast, basal levels of ROS were not affected by the age of the donor (Fig 1b). This is because DCF-DA, the probe used in our analysis, fluoresces on exposure to oxidants, especially H₂O₂ and peroxides. However, immediately upon exposure to BSO, a significant increase in the generation of ROS could be detected in cells from both young and elderly donors, with the increase being significantly higher in T cells from the elderly (Fig 1b).

2. Age-associated increase in protein carbonylation could be mimicked in T cells from young donors, upon exposure to a pro-oxidant

As protein carbonylation is a characteristic feature of oxidative stress and has been shown to occur with advancing age in various tissues, we assessed the effect of age on protein carbonylation in T cells. Increased protein carbonylation could be detected in T cells from elderly donors when compared to those from young donors (Fig 2). Upon exposure to a mild pro-oxidant, the levels of carbonylated proteins increased significantly in T cells from young donors, mimicking levels similar to those seen under basal conditions in cells from the elderly. In contrast, pro-oxidant treatment failed to induce a similar increase in carbonylated proteins, in T cells from the elderly.

3. Exposure to pro-oxidant down-regulates ATP-independent proteasomal activity while treatment with an anti-oxidant ameliorates oxidative stress induced loss in catalytic activity

We next investigated the effect of oxidative stress on 20S proteasome-associated enzymatic activities in T cells from both young and elderly donors. Exposure to oxidative stress resulted in a significant decline in 20S proteasomal peptidyl glutamyl peptide hydrolyzing or caspase-like (Fig 3a) and chymotrypsin-like (Fig 3b) activity in T cells obtained from young donors when compared to those seen under basal conditions. Our results demonstrate that 20S chymotryptic and caspase-like activities are significantly decreased under basal condition in T cells from the elderly. However, exposure to pro-oxidant condition failed to further down-regulate proteasomal activities in cells from the elderly. Thus, the decline in 20S caspase-like and chymotryptic activities observed in pro-oxidant treated T cells from young donors mimicked the decline observed in T cells from elderly (Fig 3a and 3b). We further confirmed the oxidative stress-induced decline in proteasomal proteolysis occurring in T cells, by employing a novel active site probe for proteasome in Jurkat T cells. Jurkat T cells either untreated or exposed to BSO (24h) with or without NAC pretreatment, were lysed and lysates were incubated with activity profiling probe for 2 h. Jurkat cells pretreated with 5 μ M lactacystin served as specificity control. As seen in fig.3c, exposure to BSO down regulated proteasome specific activity as exemplified by β_5 and β_1 subunits, and NAC pretreatment overrode BSO-induced inhibition in activity. This is similar to the data seen in T cells obtained from young donors. Pretreatment of T cells with an anti-oxidant, NAC, prior to pro-oxidant exposure, helped override oxidative stress-induced decline in both the post-acidic and chymotryptic activities in cells from young donors and Jurkat T cells. Additionally, similar to the observation obtained following exposure to the pro-oxidant, neither NAC nor a combination of NAC and BSO treatment induced any significant change in the 20S proteasomal activities in T cells obtained from elderly donors (Fig 3).

4. Exposure to pro-oxidant down regulates catalytic activities associated with the 26S proteasome

Previous studies from our laboratory have demonstrated significant decrease in proteasomal catalytic activity in T cells from elderly donors. However, the role of oxidative stress in the regulation of 26S proteasome in human T lymphocytes remains largely unknown. We therefore investigated the effect of pro-oxidant exposure on ATP-stimulatable enzymatic activities associated with 26S proteasome in T cells from both young and elderly human donors. Interestingly, while T cells from young donors demonstrated robust proteasomal proteolytic activity under basal conditions, exposure to the pro-oxidant, demonstrated significant loss in chymotryptic (Fig 4a) and caspase-like hydrolyzing activities (Fig 4b) similar to that observed under basal conditions in cells from elderly donors. However, it is interesting to note that the trypsin-like activity in T cells from young donors was resistant to oxidative stress-mediated decline in activity (Fig 4c). In contrast, basal levels of all the three major catalytic activities were significantly down-regulated in T cells from the elderly, the extent of decline in function

was maximum for the chymotryptic activity (69%), with caspase-like hydrolyzing and tryptic activity demonstrating between 40–50% loss in activity when compared to that observed in untreated T cells from young donors (Fig 4a, 4b and 4c respectively). However, exposure to pro-oxidant failed to further down regulate any of the ATP-stimulatable proteasomal activities in T cells from elderly donors (Fig 4).

5. Oxidative stress down regulates TNF- α -induced degradation of I κ B α

As previous studies from our laboratory have clearly demonstrated a vital role for the functional loss of 26S proteasomal activity in age-associated decline in NF κ B induction in T cells, we examined the effect of pro-oxidant treatment on activation-induced degradation of I κ B α (inhibitor of NF κ B), as a direct correlate of 26S proteasome function. Treatment with TNF- α resulted in lower levels of I κ B α when compared to untreated controls, indicating activation-induced degradation in T cells from young, but not in cells obtained from the elderly donors (Fig 5a, b), consistent with our previously published results [7]. However, upon pro-oxidant exposure, TNF- α treatment failed to induce degradation of I κ B α , mimicking lowered degradation observed in T cells from the elderly (Fig 5a, b). Interestingly, pro-oxidant exposure failed to impact on I κ B α levels following TNF- α activation in T cells from the elderly (Fig 5).

6. Pro-oxidant treatment down regulates NF κ B-dependent transcriptional activity

To further analyze whether the observed decrease in I κ B α degradation following exposure to the pro-oxidant impacts on transcriptional activity of NF κ B, we next assayed NF κ B-dependent transcriptional activity using a NF κ B-driven reporter assay. As shown in Fig. 6, pro-oxidant treatment significantly decreased TNF- α -induced NF κ B-dependent transcriptional activity by 52%, when compared to the activity in cells treated with TNF- α alone. Pretreatment with NAC inhibited the pro-oxidant-mediated loss in transcriptional activity. It should be noted that treatment with pro-oxidant alone in the absence of any stimulus consistently induced NF κ B-dependent transcriptional activity at relatively low levels (Fig 6). Treatment with anti-oxidant prior to TNF- α treatment suppressed NF κ B-dependent transcriptional activity, suggesting that TNF- α mediated induction of NF κ B transcriptional activity involves the participation of oxidant sensitive “steps” along the pathway. In fact, recent studies have demonstrated a central role for oxidant in TNF- α mediated induction of NF κ B [37].

7. Pro-oxidant treatment down regulates activation-induced T cell proliferation

We next assessed the effect of pro-oxidant treatment on T cell functional response by assaying the proliferative potential of T cells to mitogenic stimulation. As shown in Fig 7, exposure to pro-oxidant, prior to activation, decreased the proliferative ability of T cells from young donors by 40%, when compared to control cultures similarly activated in the absence of pro-oxidant, mimicking responses seen upon mitogenic treatment of T cells from the elderly. Exposure to pro-oxidant had no further dampening effect on the proliferative ability of T cells obtained from the elderly. Thus, T cells from young donors, when exposed to pro-oxidant stimuli, demonstrated a loss in proliferative potential mimicking responses observed in activated T cells from the elderly.

Discussion

As aging appears to be inherently associated with oxidative stress, an important role for ROS is becoming increasingly appreciated in T cell hypo-responsiveness during senescence. Oxidative stress-induced modifications in proteins have been documented in different cell types [21,22,38]. Consistent with this observation, our results demonstrate that increased protein carbonylation seen in T lymphocytes from elderly donors could be mimicked in T cells from young donors following exposure to pro-oxidant. As the proteasome is largely implicated in the removal of oxidized proteins [18–22,39], accumulation of carbonylated proteins during

aging and following pro-oxidant exposure suggests that proteasome may itself be a target of oxidative stress. Indeed, cross-linked proteins formed by prolonged oxidation and/or HNE modifications have previously been shown to inhibit the proteasome [16]. In addition, recent studies have demonstrated that proteasome is susceptible to free radical-induced modification and inactivation during coronary occlusion/reperfusion [21]. These results highlight an interesting paradox; the protease responsible for the removal of oxidized proteins is itself a target of oxidative modification. Thus, this study was undertaken to investigate the effect of oxidative stress-induced alteration in proteasomal activity as one of the possible mechanisms that contribute to age-associated T cell dysfunction.

We demonstrate here that exposure of T cells from young donors to pro-oxidant conditions, such as acute treatment with BSO, results in the loss of proteasomal activities that impact T cell function, mimicking hypo- responsiveness observed in T cells from the elderly. Thus, it appears that oxidative stress-mediated defects in proteasome function may well contribute to age-associated defects in T cell responses. Further, pretreatment with an anti-oxidant, NAC, afforded protection against oxidative stress-induced loss in the catalytic activity associated with 20S proteasome in the young, but not in the elderly. NAC pretreatment failed to afford complete protection of the 26S proteasome, as seen in the reporter assays, suggesting that the regulatory subunits of the 19S may be highly sensitive targets of such oxidative insults during aging. Additionally, failure of NAC to completely override proteasomal defects in T cells from the elderly as opposed to T cells from young donors exposed to pro-oxidant may indicate that acute exposure, but not chronic exposure to oxidative stress, is reversible. Alternatively, it may indicate that short term treatment with NAC, is not sufficient to override the loss induced by chronic exposure to oxidants. Additionally, it should be noted that 5mM NAC employed in our assays is capable of partial restoration of pro-oxidant-mediated effects, but higher concentration of NAC effectively inhibits 26S proteasome. Previous studies have implicated a role for NAC in modulating 19S rather than 20S catalytic subunits [40]. However, further studies are needed to directly demonstrate 19S as targets of NAC treatment. It has also been suggested that NAC may interfere with 26S activity by modifying ubiquitination of targets directed to the proteasome by modulating cellular thiol levels. However, this may not be the case in our study since we employed fluorogenic substrates that do not require ubiquitination for degradation, but nevertheless, the lack of effect on 20S activity as opposed to 26S mediated hydrolysis implicates a role for ATPase domains of 19S as targets of inhibition by NAC.

Diminished clearance of oxidized proteins during aging and age-associated diseases underscores the importance of proteasomes in the maintenance of cellular homeostasis. Results presented in this paper confirm that both ATP-stimulatable as well as ATP-independent proteasomal catalytic activities are compromised during human aging, with chymotrypsin-like activity being most affected in T lymphocytes. These results are in agreement with studies reported employing muscle lysates from LOU rat [20], but, in contrast, to the results obtained in the liver by Shibatani and coworkers, who found that basal peptidase activities were not affected, while SDS-activated 20S chymotrypsin-like and trypsin-like activities were increased and caspase-like activity was decreased with advancing age [41]. This discrepancy in data may be attributable to the specificity of the tissues employed in the studies and to the relative stoichiometry of constitutive and immuno-proteasome subunits within the catalytic core of the proteasome in these tissues.

Oxidatively modified proteins have been demonstrated to be preferentially degraded *in vitro* by the 20S proteasome in an ATP-independent fashion [14,15,20,39]. While earlier studies employing fibroblasts have suggested that the 20S proteasome might be resistant to age-associated or oxidative stress-induced modifications [18,42] recent studies employing cardiac muscles and liver from aged rodent cohorts, demonstrate significant decline in 20S proteasomal activities [21,43]. Our current results with 20S proteasomes preparations which demonstrate

chymotrypsin-like activity to be most sensitive to age-related modifications is in line with our previously reported observation obtained with 26S proteasomes, as well as, data reported by Carrard *et al*, employing peripheral blood lymphocytes from human subjects [4,22]. Studies by Carrard *et al* have also demonstrated that β -subunits of 20S proteasome are prone to glycation, HNE modification and ubiquitination during aging [22], with the β_7 and the β_{5i} subunits being the most sensitive targets for such modifications. Incidentally, the chymotryptic activity is mediated by the β_5 subunit in the proteasome, which is replaced by the β_{5i} subunit in the immuno-proteasome [11]. Hence, it would not be far fetched to speculate that the observed loss in the chymotrypsin-like activity of the proteasome during oxidative stress and aging may well be attributed to such modifications in T lymphocytes. Furthermore, it is likely, that depending on the constitution of its catalytic core, i.e. the ratio of immuno-proteasome to constitutive subunits, susceptibility to exogenous modifiers may well vary. Recent studies by Chondrogianni *et al* have provided proof of this concept in primary IMR90 human fibroblasts over-expressing β_{5i} catalytic subunits [24].

Pro-oxidant exposure of T cells from young donors via depletion of intracellular GSH resulted in diminished ATP-dependent and independent proteasomal catalytic activities, demonstrating their susceptibility to the altered cellular redox status. While the pro-oxidant treatment resulted in a significant decline in ATP-independent proteasomal activity, it was relatively less sensitive than ATP-dependent catalytic activities, and is consistent with previous studies employing fibroblasts [42].

As activation-induced degradation of I κ B α not only serves as an ideal target for regulated turn-over by the proteasome, but is also a critical step in the induction of the transcription factor NF κ B, quintessential immune response gene inducer [44]. We therefore analyzed activation-induced I κ B α degradation in T cells exposed to oxidative stress. Oxidative stress-induced decrease in proteasomal activity clearly inhibited the degradation of I κ B α , thus mimicking age-associated loss in I κ B α degradation reported previously [4,7]. This decrease in I κ B α degradation, translated into a lower transcriptional activity mediated by NF κ B, indicating that oxidative stress-mediated impairment of proteasomal activity impacts on regulatory functions dependent on the proteasome. Although activity mediated by NF κ B has been demonstrated to be susceptible to alterations in the intracellular redox status in other cell types, the role of redox remains controversial in T cells [45]. Induction of NF κ B activity has been reported in T cells following short-term administration of exogenous ROS [46] by a proteasome independent pathway. Consistent with our observations in pro-oxidant treated ILU-18 cells, chronic oxidative conditions have been shown to decrease NF κ B induction in human T cells [47]. As the proteasome is a key regulator of NF κ B activity [48], lowered levels of this transcription factor under oxidative conditions may well be due to a decrease in proteasomal activity under such circumstances.

Decline in T cell proliferation following polyclonal activation during aging and oxidative stress may be linked to decreased proteasomal catalytic activities under these conditions. This observation is consistent with the fact that proteasomes play a key role in cell proliferation by regulating the progression of cells from G₀ to S phase [49]. Additionally, several targets within the signaling cascade including PLC γ 1, intracellular Ca²⁺, IKK [50,51], as well as lower GSH [52], may contribute to lowered proliferation seen upon pro-oxidant exposure.

Thus, results presented here clearly underscore the central role of oxidative stress in decreased proteasomal proteolytic activity during aging, which may be a contributing factor for T cell hypo-responsiveness. Our results not only corroborate recent studies suggesting that altered redox status of T lymphocytes govern their responses to different stimuli [28,29], but also provide a mechanistic explanation for age-associated loss in T cell functional responses, due to lowered NF κ B induction. The finding that oxidative stress may be a forerunner for loss in

proteasomal function during aging has far reaching implications, not only for immune response gene induction but also for other regulatory processes, such as cell cycle regulation, as well as, the generation of antigenic peptides, which are important for immune regulation. Thus, interventions that override chronic oxidative stress-mediated alterations in proteasome structure and function or approaches that induce proteasomal subunits may provide valuable information for designing immunomodulatory agents for reversing senescence-induced changes in the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMC	amido-methyl-coumarin
β-NA	beta-naphthylamide
BSO	Buthionine Sulfoximine
GSH	

glutathione

IL-2R

Interleukin-2 Receptor

NAC

N-acetyl-L-cysteine

ROS

reactive oxygen species

Fig 1. Treatment with BSO depletes intracellular GSH and induces generation of ROS in T lymphocytes from young donors

A: T cells from young (Y) and elderly (O) donors were either exposed to 200 μ M BSO (+) for 24 h or left untreated (-). GSH levels were measured as described, from triplicate cultures. Results are presented as mean fluorescence intensity (MFI) and are representative of a minimum of 5 independent experiments.

B: T cells from young and elderly donors were loaded with 10 μ M DCHF-DA at 37°C for 30 min in the dark. At the end of the incubation, cells were washed and resuspended in PBS. Generation of ROS was detected at different time points, following addition of BSO, using a spectrofluorometer (excitation λ 480 nm and emission λ 525nm) and values are expressed as MFI. * Denotes statistical significance at $p < 0.001$, between the groups, as indicated.

Fig 2. Increased protein carbonylation during oxidative stress and aging

A: T cells from young (Y) and elderly (O) donors were either exposed to 200 μ M BSO for 24 h (+) or left untreated (-). Cytosolic lysates obtained from these cells were derivatized using 10 mM DNPH, and subjected to SDS-PAGE, followed by Western blotting using anti-DNP antibody and detected by ECL. Data obtained from a representative donor pair is presented.

B: Mean integrated density $\pm$ S.E of total carbonylated proteins obtained by densitometry scanning from 8 independent experiments is presented. * Denotes significance at $p < 0.05$, between the groups indicated.

B**20S peptidyl glutamyl peptide hydrolyzing activity** **C****JURKAT CELLS** $\beta 2, \beta 2i$ $\beta 5, \beta 5i$ $\beta 1, \beta 1i$ β -Actin**BSO(200 μ M)****NAC (5mM)****Lactacystin(5 μ M)**

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Fig 3. Treatment with anti-oxidant ameliorates pro-oxidant-mediated decline in 20S proteasomal activities, but fails to restore age-associated loss in activity

A & B. T cells (50×10^6) from young (Y) and elderly (O) donors were either pretreated with 30 mM NAC for 2 h or left untreated (–) for 24 h. At the end of pretreatment cells were exposed to 200 μ M BSO (+). Enriched 20S proteasome fractions from these cells, (50 μ g) were used in proteolytic activity assay evaluated using specific fluorogenic substrates, Suc-LLVY-AMC and N-LLE- β NA for chymotryptic and post-acidic activity, respectively. Data are presented as specific activity/mg/hr $\pm$ SE obtained from a minimum of 5 donor pairs. * Denotes statistical significance at $p < 0.001$, between the groups indicated. **Fig.3.A:** Represents results of chymotryptic activity and **Fig.3.B:** represents results of peptidyl glutamyl hydrolytic activity.

C. BSO down regulates 20S proteasome activity in Jurkat T cells assayed employing a novel biotinylated proteasome active site probe. Lysates obtained from untreated Jurkat cells, or those exposed to either 200 μ M BSO for 24 h or pretreated with NAC (5mM) for 2h followed by treatment with BSO for 24h, were labeled with 5 μ M biotinylated proteasome – profiling probe for 2h at 37 C. Labeled lysates were resolved using SDS-PAGE on 12% gels and immunoblotted using streptavidin conjugated to horse-radish peroxidase and ECL. Lysates obtained from cells treated with 5 μ M Lactacystin (Lacta.) for 2 h, prior to labeling with the active site probe, is included as specificity control.

B 26S peptidyl glutamyl peptide hydrolyzing activity

Fig 4. 26S proteasome associated catalytic activities are down regulated in T cells from the elderly and following exposure to pro-oxidant treatment

T cells (50×10^6) from young (Y) and elderly (O) donors were exposed to 200 μ M BSO (+) or left untreated (-) for 24 h. At the end of treatment, 50 μ g of enriched 26S proteasome fraction were assayed for chymotryptic activity (A); peptidyl glutamyl hydrolytic activity (B); and tryptic activity (C), using, Suc-LLVY-AMC, N-LLE- β NA, Bz-R-AMC; respectively. Data are presented as specific activity/mg/hr $\pm$ SE obtained from a minimum of 5 donor pairs. * Denotes statistical significance at $p < 0.001$, between the groups indicated.

Fig 5. TNF- α mediated degradation of I κ B- α is affected by aging and pro-oxidant treatment

A: T cells from young (Y) and elderly (O) donors were either exposed to 200 μ M BSO (+) for 24 h or left untreated (-). At the end of 24 h, cells were either treated with 10 ng/ml TNF- α for 10 min (+) or left without treatment (-). Cytosolic lysates were equalized for protein (30 μ g) and subjected to SDS-PAGE, followed by Western Blotting using antibody to I κ B α and detected by ECL. Data obtained from a representative donor pair is presented. The blot was stripped and probed with antibody to β -actin to demonstrate equal protein loading in all the lanes.

B: Quantitation of I κ B α degradation as detected by the disappearance of the specific band in Western blots was carried out by densitometry scanning, from 7 independent donor pairs.

C: Degradation of I κ B α is presented as percent decrease in levels obtained in TNF- α treated cells when compared to untreated controls. Values represent Mean $\pm$ SE of percent degradation obtained from independently conducted experiments 7 donor pairs.

* Denotes statistical significance at $p < 0.001$, between the indicated groups.

Fig 6. Pretreatment with pro-oxidant down-regulates NFκB-dependent reporter activity induced by TNF-α

ILU-18 cells were either exposed to 200 μM BSO (+) for 24 h or left untreated (-). At the end of 24 h, the cells were activated with 10 ng/ml of TNF-α for 24 h (+), or left untreated (-). To override the effect of pro-oxidant exposure; cells were also pretreated for 2 h with NAC, followed by BSO and TNF-α as indicated. Luciferase reporter assay was performed to determine NFκB-dependent transcriptional activity, as per manufacturer's protocol. * Denotes statistical significance at $p < 0.001$ between indicated groups.

Fig7. Aging and oxidative stress down-regulates activation-induced T cell proliferation

T cells from young (Y) and elderly (O) donors were either exposed to 200 μM BSO (+) for 24 h or left untreated (-). At the end of incubation, cells (1×10^6) were stimulated with plate-bound 1 μg/ml anti-(CD3+ CD28) for 48 h at 37°C. Proliferation was measured by ^3H -thymidine uptake for the last 6 h of culture. Data are presented as proliferative index $\pm$ SE from 8 independent experiments. * Denotes statistical significance at $p < 0.001$, between groups.