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RESEARCH ARTICLE

Molecular insights into the unique properties of the blood-circulating proteasome

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Abstract
Proteasomes are essential for protein degradation and maintaining cellular balance, yet their roles in extracellular fluids are not well understood. Our study investigates the freely circulating proteasome in blood, to uncover its unique molecular characteristics, compared to its intracellular counterparts. Using a transgenic mouse model, mass spectrometry, and biochemical tools, we show that the predominant proteasome in serum is the free uncapped 20S particle, which seems to assemble intracellularly before entering the bloodstream. This serum proteasome is composed of constitutive and immuno subunits and exhibits all three catalytic activities. Moreover, the complex displays distinct post-translational modifications, indicating specialization for extracellular roles, as demonstrated by its enhanced caspase-like activity. We also found that physiological stress significantly upregulates serum 20S proteasome levels, paralleling human data. This research highlights the specialized characteristics of circulating proteasomes, offering new insights into protein turnover in the blood with significant implications for understanding proteostasis beyond the intracellular environment.

KEYWORDS
blood, extracellular, Mass Spectrometry, plasma, Proteasome

1 | INTRODUCTION

Proteasomes are essential players in proteostasis, degrading regulatory, redundant, and damaged proteins to maintain cellular function (Ciechanover, 2017; Schmidt & Finley, 2014). While traditionally viewed as intracellular complexes, evidence shows that they also exist in the extracellular space (Ben-Nissan et al., 2022), including in extracellular vesicles (EVs) (Bonhoure et al., 2022; Dieudé et al., 2015), cerebrospinal fluid (Mueller et al., 2012), bronchoalveolar lavage (Sixt et al., 2007), epididymal fluid (Tengowski et al., 2007) and plasma (Dwivedi et al., 2021). Our recent analysis of 143 proteomic datasets revealed proteasome subunits in 25 extracellular fluids, such as tears, sweat, pericardial, seminal, and amniotic fluids (Ben-Nissan et al., 2022). This suggests that proteasome-mediated protein quality control operates in the extracellular environment. However, understanding of extracellular proteasomes lags behind that of their intracellular counterparts, and little is known about this fluid-based degradation system. In this study, we focused on extracellular proteasomes circulating in the blood.

Proteasome-mediated degradation occurs via two pathways: one ubiquitin- and (adenosine triphosphate) ATP-independent, and the other dependent on both (Goldberg, 2003; Kumar Deshmukh et al., 2019). The 20S proteasome complex, responsible for ubiquitin- and ATP-independent degradation, targets proteins with unfolded or unstructured regions (Ben-Nissan & Sharon, 2014; Kumar Deshmukh et al., 2019). It consists of 28 subunits arranged into a cylindrical structure with two outer α-rings

(PSMA1–PSMA7) and two inner β-rings (PSMB1–PSMB7), housing three types of proteolytic sites: chymotrypsin-like (PSMB5), caspase-like (PSMB6) and trypsin-like (PSMB7) activities (Kish-Trier & Hill, 2013). Immunoproteasomes are formed when these subunits are replaced by their counterparts: PSMB8, PSMB9, and PSMB10 (Ferrington & Gregerson, 2012). The 20S complex can be capped by activators like PA28$\alpha\beta$, PA28γ, or PA200, which open the gate and enhance catalytic activity (Cascio, 2021; Pickering & Davies, 2012). For ubiquitin-dependent degradation, the 20S proteasome associates with one or two 19S regulatory complexes to form the 26S or 30S proteasome (Hershko & Ciechanover, 1998). Ubiquitination requires ATP-dependent enzymes (E1, E2, E3) to tag substrates, after which the 19S complex recognizes polyubiquitin chains and facilitates substrate deubiquitination, unfolding and translocation into the 20S core, for degradation (Scheffner et al., 1995).

The first discovery of proteasomes in the blood was reported about 30 years ago in a study involving patients with hematologic malignancies (Wada et al., 1993). Since then, extensive clinical data have accumulated regarding the presence of circulating proteasomes and their link to disease progression and outcome (reviewed in Dwivedi et al. (2021)). For instance, elevated levels of circulating proteasomes have been found in the plasma of patients suffering from blood, skin and solid cancers, trauma, sepsis, and autoimmune disorders, including rheumatoid arthritis and systemic lupus erythematosus (Dwivedi et al., 2021; Egerer et al., 2002). Moreover, in various diseases, a positive correlation between circulating proteasome levels, treatment efficacy and survival rates, was discovered, suggesting the involvement of this understudied proteasome complex in pathophysiology. For example, in lymphoid malignancies such as chronic lymphatic leukaemia, multiple myeloma, and non-Hodgkin's lymphoma, decreased levels of plasma 20S particles are detected in the early stages of the disease in comparison to healthy individuals, which vastly increase throughout later, more aggressive disease stages (Dwivedi et al., 2021). However, it remains unknown how similar or different these extracellular proteasomes are from their intracellular counterparts.

In our investigation, we utilized advanced mass spectrometry (MS)-based methodologies, biochemical tools, and mouse models to explore the architecture, composition, and molecular properties of the circulating proteasome complex. Our results revealed that the free uncapped 20S particle is the primary circulating proteasome component in serum (c20S). We found no evidence for proteasome assembly chaperones in serum, suggesting that c20S complexes undergo intracellular biogenesis before being transported to the blood. Both the constitutive and immuno c20S proteasomes are present in serum, with immunoproteasome subunits notably enriched compared to their levels in liver proteasomes. While these proteasomes exhibit all three catalytic activities, the c20S displayed enhanced caspase-like activity and reduced trypsin- and chymotrypsin-like activities, compared to the intracellular liver proteasome. Furthermore, we identified distinct modifications on the c20S, indicating that these complexes are adapted for their extracellular role. We also demonstrated that changes in physiological conditions lead to a significant increase in c20S proteasome levels in mice, mirroring observations in humans, and providing an experimentally tractable model system for further investigation of c20S proteasomes. These findings highlight the unique nature of circulating proteasomes and pave the way for future research in this emerging field.

2 | METHODS

2.1 | Mice strains

Unless indicated otherwise, all mice used in this study were Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-modified C57Bl/6JOlaHsd mice, bearing a chromosomal insertion of a C-terminal FLAG tag in the PSMB2 gene. Ethics statement—all animal studies were conducted in accordance with the regulations formulated by the Institutional Animal Care and Use Committee (IACUC; protocol # 05560621-1).

A detailed description of the methods used in this study can be found in the Supplementary Material.

3 | RESULTS

3.1 | Generation of a transgenic mouse model expressing FLAG-tagged 20S proteasome

Cell line-based studies, although crucial for elucidating intracellular protein degradation pathways, particularly the 26S proteasome-ubiquitin pathway, are not suitable for serum compartment studies. Therefore, to investigate the circulating proteasome system, we generated a transgenic mouse model expressing a FLAG-tagged 20S proteasome. We selected the PSMB2 subunit for tagging because of its prior success in single-step 20S proteasome purification from yeast and HEK293 cells (Ben-Nissan et al., 2019; Verma et al., 2000). Using CRISPR, we inserted a FLAG tag at the C-terminus of the PSMB2 subunit of the 20S proteasome, spaced by a short linker. Genotyping and chromosomal sequencing confirmed the FLAG tag's correct insertion within the PSMB2 reading frame (Figure S1A,B). Western blot analyses showed the presence of one and two copies of the edited PSMB2 protein in homozygous and heterozygous transgenes, respectively (Figure S1C). The generated mice are viable and show no visible differences from wild-type (WT) mice.
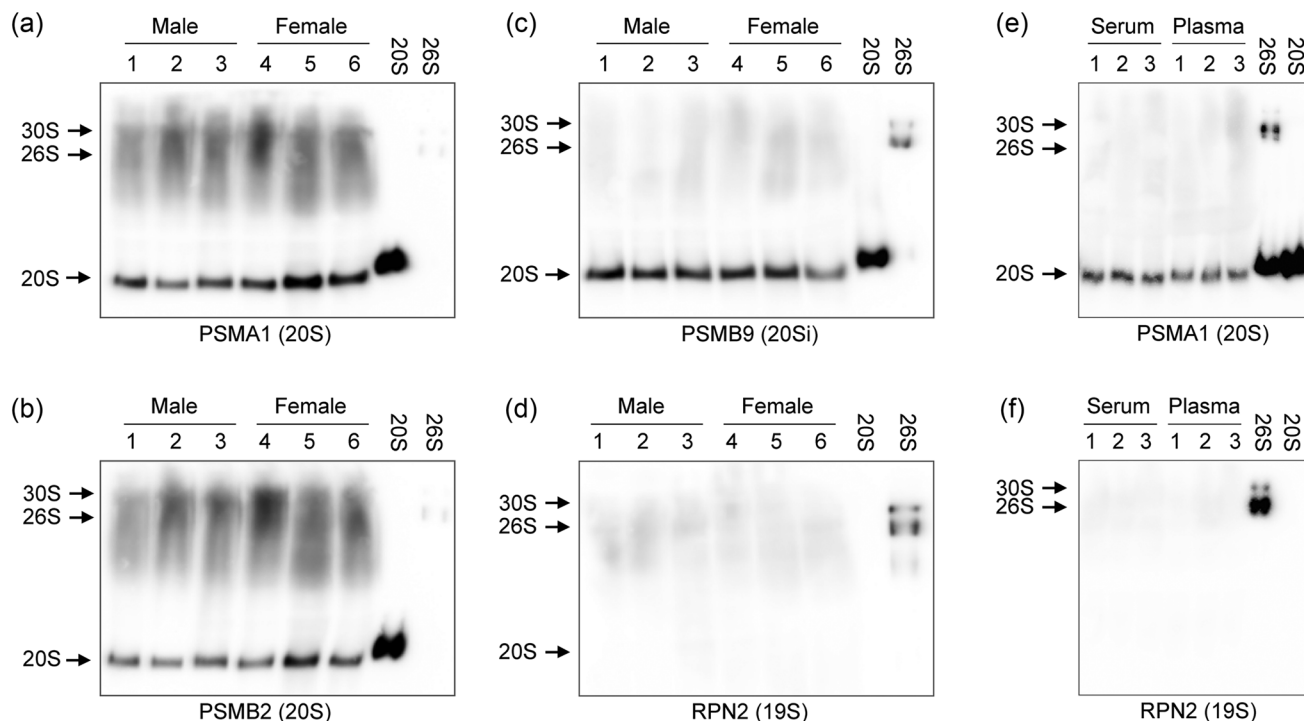
FIGURE 1 Blood contains primarily intact and uncapped c20S proteasomes. Sera from three male and three female mice were separated on native gels and subjected to Western blot analysis using antibodies against the 20S proteasome α-subunit PSMA1 (a), the β-subunit PSMB2 (b), the immunoproteasome subunit PSMB9 (c), and the 19S regulatory particle subunit RPN2 (d). The antibodies detected only the intact c20S proteasome, with no larger proteasome species identified. Purified rat 20S and 26S proteasomes were used as positive controls. (e) A comparison between serum and plasma, collected from the same animals, showed that both blood fluids contain similar amounts of c20S proteasomes, but no higher molecular weight proteasome subtypes. (f) An antibody against RPN2, a 19S proteasome subunit, further confirmed that both fluids are devoid of 26S proteasomes.

3.2 │ Dominance of the 20S proteasome subtype in blood

Earlier studies have indicated that proteasomes in the bloodstream predominantly exist as the uncapped 20S catalytic particle (Majetschak et al., 2010; Yun et al., 2020; Zoeger et al., 2006). However, proteomic analyses of blood samples have also detected subunits of the 19S proteasome and other regulatory particles (Dey et al., 2019; Harel et al., 2015; Liu et al., 2018; Park et al., 2020), suggesting that the circulating 20S proteasome may associate with these regulators. To investigate the repertoire of proteasome subtypes in blood, we separated sera from 6 to 10 week-old male and female mice, using native gels in the presence of ATP, to preserve the integrity of potential capped proteasome particles. The native gels were then subjected to Western blot analyses with various proteasome antibodies (Figure 1).

Antibodies against both α- and β-subunits (PSMA1 and PSMB2, respectively), identified the c20S proteasome, which migrated alongside purified 20S proteasomes. These antibodies did not specifically label any higher molecular weight species that correspond to 26S or other capped proteasomes (Figure 1a,b). Notably, the smeary signals of higher molecular weight species in these blots were non-specific, as validated by affinity purification of the complex (Figure S2A). Blotting against PSMB9 also detected the presence of immunoproteasomes, specifically in the form of free 20S particles (20Si) (Figure 1c and Figure S2B). Similarly, antibodies against RPN2, one of the subunits of the 19S regulator, did not detect any noticeable 26S proteasome species in the separated sera (Figure 1d). The results were consistent regardless of the sex of the mice. In contrast, analysis of mouse liver lysates detected both 20S and 26S proteasomes (Figure S3). These findings suggest that, unlike the intracellular proteasome population, the 20S particle is the major proteasome subtype in the blood.

Given the considerable variability in reported 20S proteasome levels between serum and plasma (Choi et al., 2021; Dwivedi et al., 2021), we decided to compare these levels directly in samples obtained from the same animals. This comparison aimed to better understand the relative abundance of 20S proteasomes in both blood fluids and to ensure that the absence of the 26S proteasome in serum was not due to clotting (Figure 1e,f). Comparative analysis of Western blots using antibodies against PSMA1 revealed only marginal differences between the levels of the c20S in the two blood fluids, with slightly higher levels of c20S in serum. Similarly, no specific signals corresponding to the 26S proteasome, free 19S regulatory particles, or other capped proteasomes were identified in either serum or plasma.

Lastly, to confirm that the 20S proteasome is indeed circulating freely in the serum, rather than being encapsulated within, or associated with EV membranes, we conducted additional analyses. We performed FLAG-affinity purification, reasoning that freely circulating proteasomes will be captured by the anti-FLAG beads, while EV-encapsulated/associated species will remain in the flow-through. The results indicated that the majority of serum c20S was captured by the FLAG beads, confirming its free circulation in the blood. Only a minor fraction flowed through the beads, suggesting it is associated with serum EVs, as indicated by the presence of the EV markers TSG101 and Alix (Figure S2B). These findings confirm that freely circulating 20S particles are the predominant species in the blood.

3.3 | Circulating proteasomes do not assemble in the blood

To gain a deeper understanding of the repertoire of proteasomes and proteasome-associated proteins in blood, we performed FLAG-affinity purification of proteasomes from mouse serum and livers under conditions that maintain the integrity of 26S proteasomes. Purified serum and liver samples, collected from the same animals, were then subjected to proteomic analysis by liquid chromatography (LC)-MS/MS (Table S1).

As expected, liver 20S proteasomes co-purified with all 19S proteasome subunits, as well as with the known proteasome regulators, including PSME1-PSME4 and PSMF1, the proteasome assembly chaperones PSMG1 and PSMG2, and the proteasome maturation protein POMP1 (Table S1). In contrast, the circulating 20S proteasome from blood did not co-purify with any regulator subunits or chaperone proteins. These findings further support our observation that blood is predominantly devoid of capped proteasomes. Moreover, the absence of assembly chaperones suggests that the assembly of the proteasome complex does not occur within the blood, but rather the proteasome is transported there from other tissues.

3.4 | Circulating 20S proteasomes are enriched with immunoproteasome subunits

The proteomic analysis further revealed that c20S proteasomes, like intracellular liver 20S proteasomes, contain the three constitutive catalytic subunits (PSMB5, PSMB6, PSMB7) as well as the three alternative immunoproteasome subunits (PSMB8, PSMB9, PSMB10) (Table S1). Since immunoproteasome subunits are known to be differentially expressed across tissues (Uhlén et al., 2015), we sought to examine their relative abundance in blood compared to liver. Western blot analysis using antibodies specific to both constitutive and immunoproteasomes catalytic subunits was performed on purified proteasomes from blood and liver (Figure 2). The results showed that c20S proteasomes are significantly enriched with immunoproteasome subunits compared to liver proteasomes, where the constitutive catalytic subunits are more predominant. These findings strongly suggest a significant role for immunoproteasomes in serum, paving the way for future research to explore their unique functional contributions.

3.5 | Circulating 20S proteasomes exhibit unique subunit modifications

We next used MS to examine the structural characteristics of c20S proteasomes and compare them to their intracellular liver counterparts. Native MS analysis of purified proteasomes confirmed the integrity of c20S and identified stable half-proteasome subcomplexes in both sources (Figure S4). Tandem MS (MS/MS) was performed to determine subunit composition by accurately measuring and assigning masses to monomeric subunits and dimers (Vimer et al., 2020) (Figure S5 and Table S2). High-resolution LC-MS/MS and top–down analysis of intact subunits confirmed subunit identities (Figure 3, Figures S6–S8 and Table S2). This approach provided detailed insights into c20S's unique features, including potential modifications and differences from intracellular proteasomes.

Our analysis of c20S proteasomes revealed 27 distinct subunit masses, which were present in at least three biological replicates (Table S2). Notably, nine of these masses were unique to serum (Figure 3, labeled by ovals) and were absent from all liver samples (Figures S5 and S6). The remaining 18 masses corresponded to all canonical 20S proteoforms as found in other organisms (rat, rabbit, yeast and HEK293 cells) (Vimer et al., 2020), including the three immunoproteasome subunits and two proteoforms of PSMA3 (with and without the constitutive C-terminal phosphorylation) (Figure 3 and Figure S7). Detailed examination of the unique masses uncovered several intriguing findings related to truncations, cysteinylations and glutathionylations.

We observed tissue-specific truncations despite similar purification, processing and storage procedures for both liver and blood 20S proteasomes. In serum, the C-terminal lysine residue of PSMA4 was truncated (Δ128 Da) compared to its liver counterpart. Conversely, in liver samples, the C-termini of PSMA7 and PSMB5 were truncated, losing the last two residues (SA and VP, respectively), while in blood, both subunits remained intact. These differences suggest that the observed modifications are not due to sample handling (Zivkovic et al., 2022) but rather reflect the biological context of the proteasomes in their respective environments.
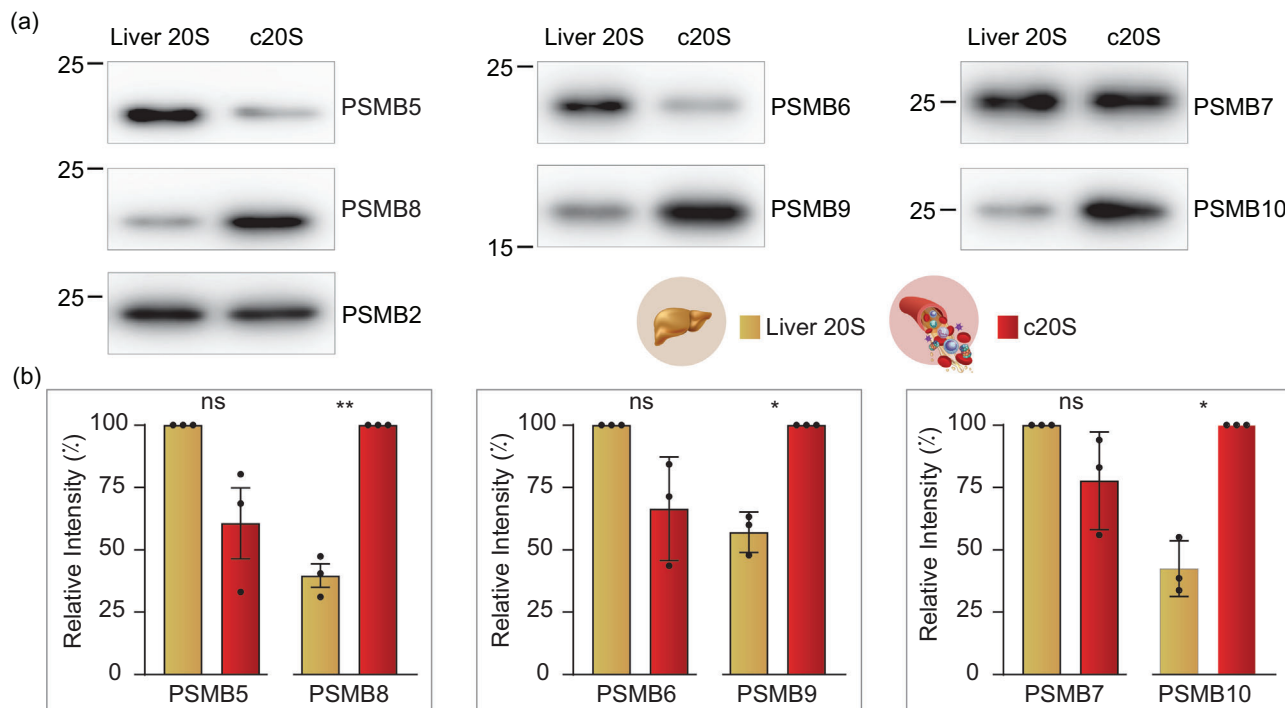
(a)

(b)

FIGURE 2 Immunoproteasome subunits are enriched in circulating 20S proteasomes. (a) Equal amounts of purified liver and c20S proteasomes were separated on SDS-PAGE, followed by Western blotting with antibodies targeting the constitutive and immunoproteasome catalytic subunits. (b) Quantification of relative expression levels, reveals a significant enrichment of immunoproteasome subunits in c20S proteasomes compared to liver proteasomes. Bars represent averages from three biological replicates, with error bars indicating SE. Values were compared by paired sample t-test. Asterisks denote p-values of 0.006 for PSMB8, 0.0117 for PSMB9, and 0.0123 for PSMB10. Expression intensities were normalized to the level of the constitutive non-catalytic subunit, PSMB2.

Six c20S subunits, including both α- and β-subunits (PSMA1, PSMA7, PSMB2, PSMB7, PSMB8 and PSMB9), displayed a mass addition of 119 Da, corresponding to cysteinylation. This post-translational modification (PTM) occurs when a cysteine molecule forms a disulfide bond with a free cysteine residue on a protein (Bechtel & Weerapana, 2017). The relative levels of the cysteinylated subunits ranged from 22% of the total pool of PSMA1 to 95% of PSMB9 (Figure 3b–g). Top-down fragmentation patterns of cysteinylated PSMA7 and PSMB9 not only identified the subunits but also pinpointed the specific cysteine residues modified (Cys63 in PSMA7 and Cys106 in the mature form of PSMB9) (Figure S8).

c20S PSMA1 and PSMB2 displayed a different mass addition of 305 Da, corresponding to glutathionylation. This is another thiol-based PTM where glutathione, a tripeptide antioxidant, forms a disulfide bond with a cysteine residue on a protein, regulating protein function (Musaogullari & Chai, 2020). This modification was detected for these two subunits at similar levels as their cysteinylated counterparts. To validate the identity of the modified 20S subunits, we compared c20S proteasomes before and after reduction, finding that both cysteinylation and glutathionylation could be reversed by reduction, supporting the identity of these thiol modifications (Figure S9).

Notably, neither cysteinylation nor glutathionylation was found in any of the biological repeats of mouse liver proteasomes (Figures S5 and S6), despite comparable intracellular and extracellular cysteine levels (Chen et al., 1990; Gaitonde, 1967; Iyer et al., 2009; Watson et al., 2020) and significantly higher intracellular glutathione levels (Anderson & Meister, 1980; Iyer et al., 2009; Roman et al., 2013; Watson et al., 2020). This suggests that the mere presence of proteasomes in an environment containing these molecules is not the primary factor for acquiring these specific modifications. Instead, the acquisition of cysteinylation and glutathionylation appears to be an active process regulated by the biological context of the proteasomes within the blood.

3.6 | Circulating c20S proteasomes are active, possessing all three catalytic functions

Having identified unique modifications in the blood-circulating 20S proteasome, we aimed to investigate whether these modifications influence the catalytic activity of the complex. Previous clinical studies reporting c20S functionality were mainly based on plate-based assays using fluorogenic peptide substrates to measure proteasome activity (Dwivedi et al., 2021). However, given that blood plasma contains various proteases involved in processes such as blood coagulation, complement activation, and fibrinolysis (Maffioli et al., 2020), there is a concern that the detected peptidase activity in such assays may not originate only from
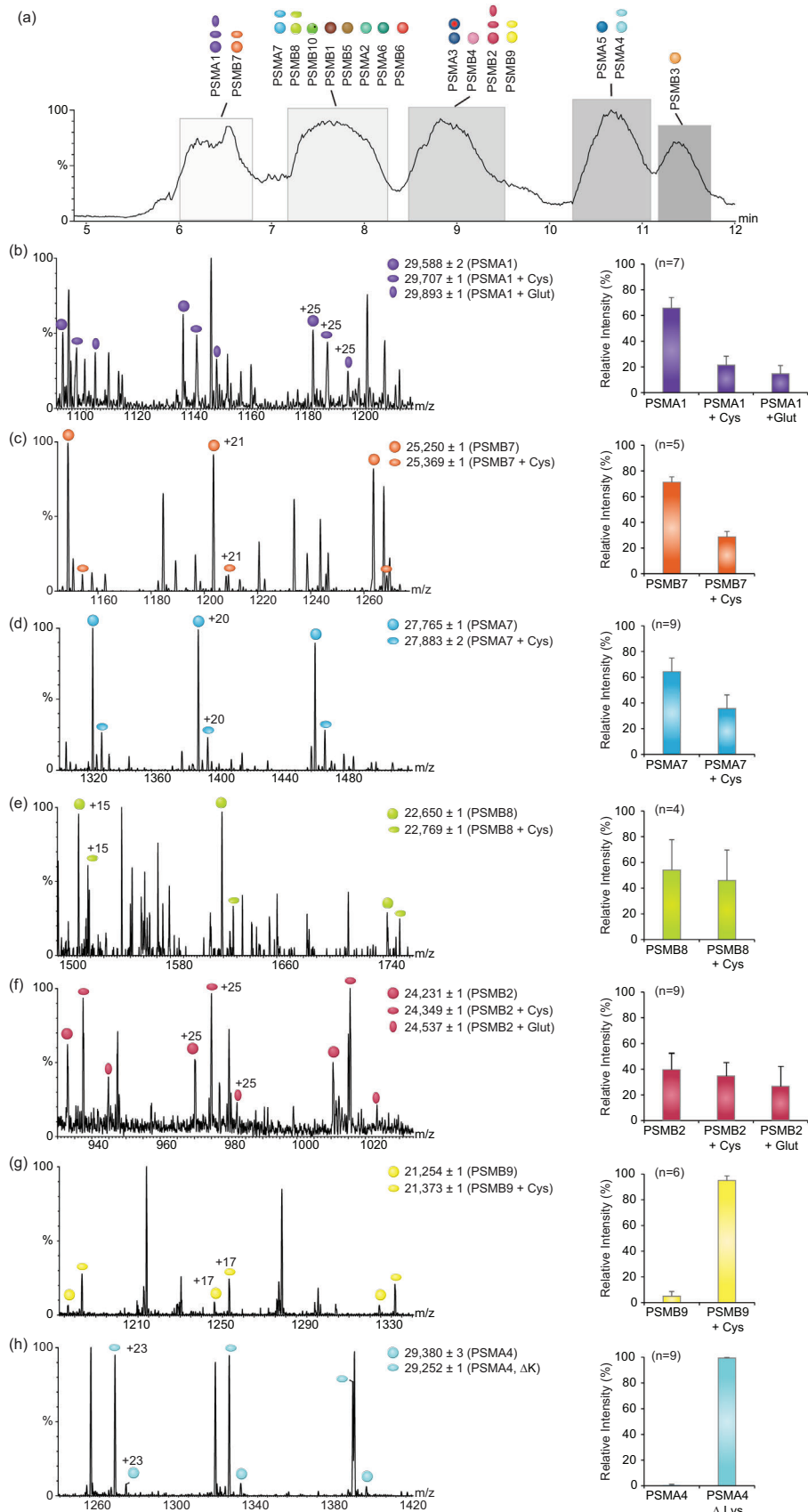
FIGURE 3 Multiple c20S proteasome subunits exhibit unique modifications, including truncations, cysteinylations, and glutathionylations. c20S proteasomes were loaded onto reversed-phase columns and eluted into a MS for accurate mass analysis. (a) A representative total ion chromatogram (using a

(Continues)

the c20S, potentially leading to false-positive results (Rodgers & Dean, 2003). Therefore, we used native gels to separate the proteasomes from other serum components, including low-molecular-weight proteases, prior to incubation with the fluorogenic peptide substrates. This allowed us to accurately monitor the specific levels and activities of c20S proteasomes in a targeted manner.

We performed in-gel activity assays on sera from 6 to 10 week old male and female mice (Figure 4a–c). The activity of the intact c20S proteasome was detected in the same position as purified proteasomes and appeared to be highly similar in both serum and plasma (Figure S10A). Both the caspase- and chymotrypsin-like activities were clearly observed in these gels, with the chymotrypsin-like activity being enhanced by SDS, as expected (Figure S10B). The proteasome inhibitor epoxomicin significantly suppressed the chymotrypsin-like activity (Figure 4d), while the overall levels of the c20S remained unchanged (Figure 4e).

The trypsin-like activity of the proteasome, on the other hand, appeared faint, prompting us to further examine the catalytic activity of purified c20S. Peptidase activity assays using the three fluorescent peptide substrates confirmed that all three catalytic proteasome subunits are active, with two of them being inhibited by the available specific inhibitors (leupeptin for the trypsin-like activity and epoxomicin for the chymotrypsin-like activity) (Bogyo & Wang, 2002) (Figure 4f, Kisselev & Goldberg, 2001), as found also in intracellular liver proteasomes (Figure S11A). This result dismissed the possibility that c20S proteasomes do not exhibit significant trypsin-like activity. The apparent low trypsin-like activity of the c20S in native activity gels (Figure 4b) is therefore attributed to the strong signals detected above the c20S band, which restrict the exposure times for visualization.

We identified two higher molecular weight bands in the native activity gels with peptidase activity (Figure 4a–d, asterisks), but they were not sensitive to epoxomicin, indicating they were not proteasomes. This was confirmed by FLAG depletion, which removed c20S activity but left these bands unaffected (Figure S10C). Western blot analysis also showed no reaction with 20S subunit antibodies (Figure 1 and Figure S2). Proteomic analysis revealed that none of the higher molecular weight bands contained 20S proteasome subunits or proteasome-related proteins such as the 19S base, lid subcomplexes, or other regulatory proteins, further confirming that the blood contains predominantly uncapped 20S proteasomes (Table S3). Instead, these bands contained proteins related to complement, coagulation, and cholesterol metabolism, along with unique proteases likely responsible for non-specific peptidase activity (Figure S12). Given the abundance of active proteases in blood, these findings highlight the importance of characterizing proteasome activity via native gels, as plate-based assays may not effectively distinguish proteasomes from other proteases.

Lastly, we examined the proteolytic activity of the purified c20S by performing degradation assays using a partially unfolded c20S proteasome substrate from blood, Galectin-3 (Figure 4g,h). Under the examined experimental conditions, Galectin-3 was not very stable, and its levels gradually decreased during the 3-h incubation period. However, when incubated together with purified c20S proteasomes, Galectin-3 degraded almost to completion within 1 h, while preincubation of the c20S with epoxomicin significantly inhibited its proteolytic activity. Similar results were found for the intracellular liver 20S proteasome (Figure S11B,C). Cumulatively, these results indicate that blood contains active 20S proteasomes.

3.7 | The c20S proteasome exhibits distinct activity compared to the liver proteasome

Having established that (I) the predominant proteasome subtype in serum is the 20S particle, and (II) this complex displays all three catalytic activities, we turned our attention to comparing its catalytic capacity with that of its intracellular counterpart in the liver. To do this, we used MV151, a fluorescent activity probe that specifically and covalently binds to the active catalytic subunits of both the proteasome and immunoproteasome (Verdoes et al., 2006), enabling us to monitor the activity of individual catalytic subunits (Turker et al., 2023).

The assay involved incubating equivalent amounts of serum and liver 20S proteasomes with MV151 over a time course, followed by SDS-PAGE separation and quantification of the fluorescent signals from the various subunits (Figure 5a). Analysis from five independent biological replicates revealed significant differences in catalytic properties between blood and liver 20S proteasomes. Specifically, c20S proteasomes exhibited a marked increase in caspase-like activity and a reduction in chymotrypsin-like activity from the constitutive PSMB5 subunit (Figure 5b). In contrast, trypsin-like activity and immuno subunit-mediated chymotrypsin-like activity showed a slight but statistically insignificant increase in liver proteasomes. These findings emphasize the distinct properties of c20S, suggesting it has undergone specialization to meet the unique biological demands of maintaining blood homeostasis.
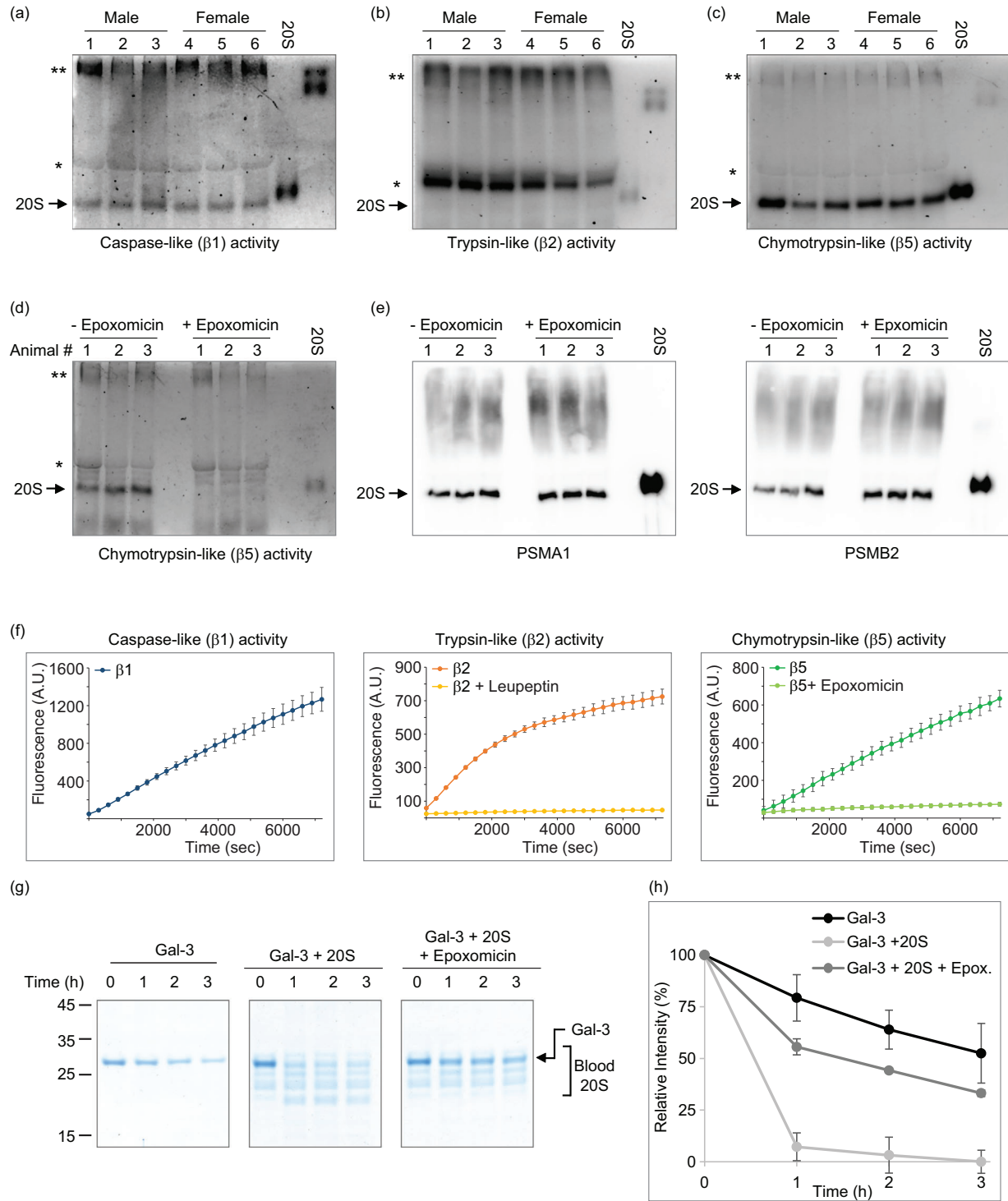
FIGURE 4 The c20S proteasome exhibits active catalytic functions. (a–c) Sera from three male and three female mice were separated on native gels and subjected to in-gel peptidase activity assays using fluorescent substrates specific to the caspase- (a), trypsin- (b), and chymotrypsin-like (c) activities of the 20S proteasome. Caspase-like and chymotrypsin-like activities were clearly detected (a, c), while trypsin-like activity was faint (b). Asterisks indicate higher molecular weight species in the serum with peptidase activity. (d) Epoxomicin inhibited the chymotrypsin-like activity of the c20S proteasome but did not affect the peptidase activity of the higher molecular weight serum components (asterisks). (e) Western blot analyses confirmed that proteasome levels remained stable during incubation, indicating that the decline in activity was not due to a reduction in c20S levels. Purified rat 20S proteasome was used as a control. (f) FLAG-affinity-purified c20S from mouse serum was subjected to peptidase activity assays in plates, using fluorescent substrates for caspase-like, trypsin-like, and chymotrypsin-like activities. Fluorescence monitored over 2 h, showed that c20S proteasomes exhibit all three catalytic activities. Leupeptin inhibited the trypsin-like activity, and epoxomicin inhibited the chymotrypsin-like activity, confirming the specific peptidase activities. (g) In-vitro degradation assays using the purified c20S and a model substrate, the partially unfolded blood protein, Galectin3 (Gal-3). The proteasome inhibitor, epoxomicin, was used as a negative control. (h) Averaged quantification of three independent experiments; error bars represent STD.
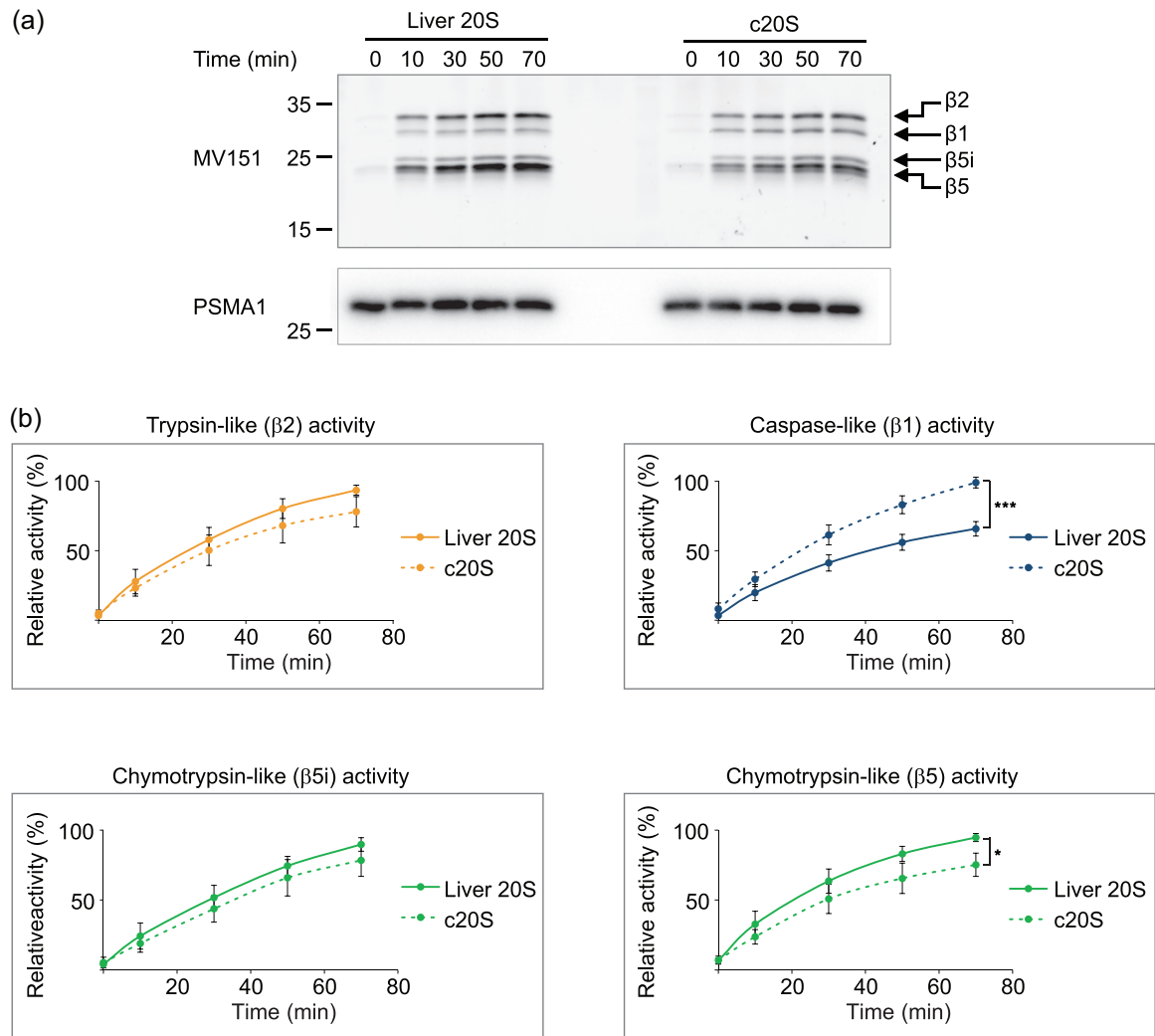
FIGURE 5 The c20S proteasome exhibits distinct activity compared to the liver proteasome. (a) FLAG affinity-purified 20S proteasomes from mouse liver and serum were subjected to in-vitro activity assays using the fluorescent activity probe, MV151. Samples were incubated with MV151 for various time points, followed by separation on SDS-PAGE and in-gel fluorescence detection. (b) Quantification of fluorescence intensity for the different catalytic subunits was performed using densitometry analysis. Values at the last time point were compared using a 2-way ANOVA, accounting for tissue and batch. Asterisks denote p-values of 0.000667 for the Caspase-like activity, and 0.0342 for the Chymotrypsin-like activity. Data represents the average of five biological replicates, error represent SE.

3.8 | Increase in c20S concentration is detected in mice under various pathological and physiological conditions

To effectively study circulating blood proteasomes, cell culture systems are insufficient, necessitating an animal model that can replicate observations made in clinical studies on humans. Numerous clinical studies over the past years have reported elevated levels of c20S proteasomes in various diseases, including different types of cancers, autoimmune disorders, trauma, and sepsis (Dwivedi et al., 2021). However, similar observations have not been replicated in mice, raising the question of whether mice, like humans, exhibit an increase in circulating proteasome levels under disease conditions. To address this gap and determine if mice can serve as a suitable model for studying circulating proteasomes in disease, we conducted experiments to investigate c20S levels in mice under various pathological conditions.

We began by examining the blood of both healthy and diseased mice suffering from ovarian cancer, a condition where elevated levels of proteasomes have been reported in the blood of women (Heubner et al., 2011). Sera were collected from five healthy female mice and six female mice with high-grade serous carcinoma (Iyer et al., 2021), which were sacrificed 6 weeks after intraperitoneal injection of PPNM cells. The sera were separated on native gels and analyzed by peptidase assays and Western blots (Figure 6). As shown in Figure 6a,c, sera from the diseased mice exhibited significantly higher levels of 20S proteasomes. Immunoproteasome levels also increased, although to a lesser extent than constitutive proteasomes. Correspondingly,
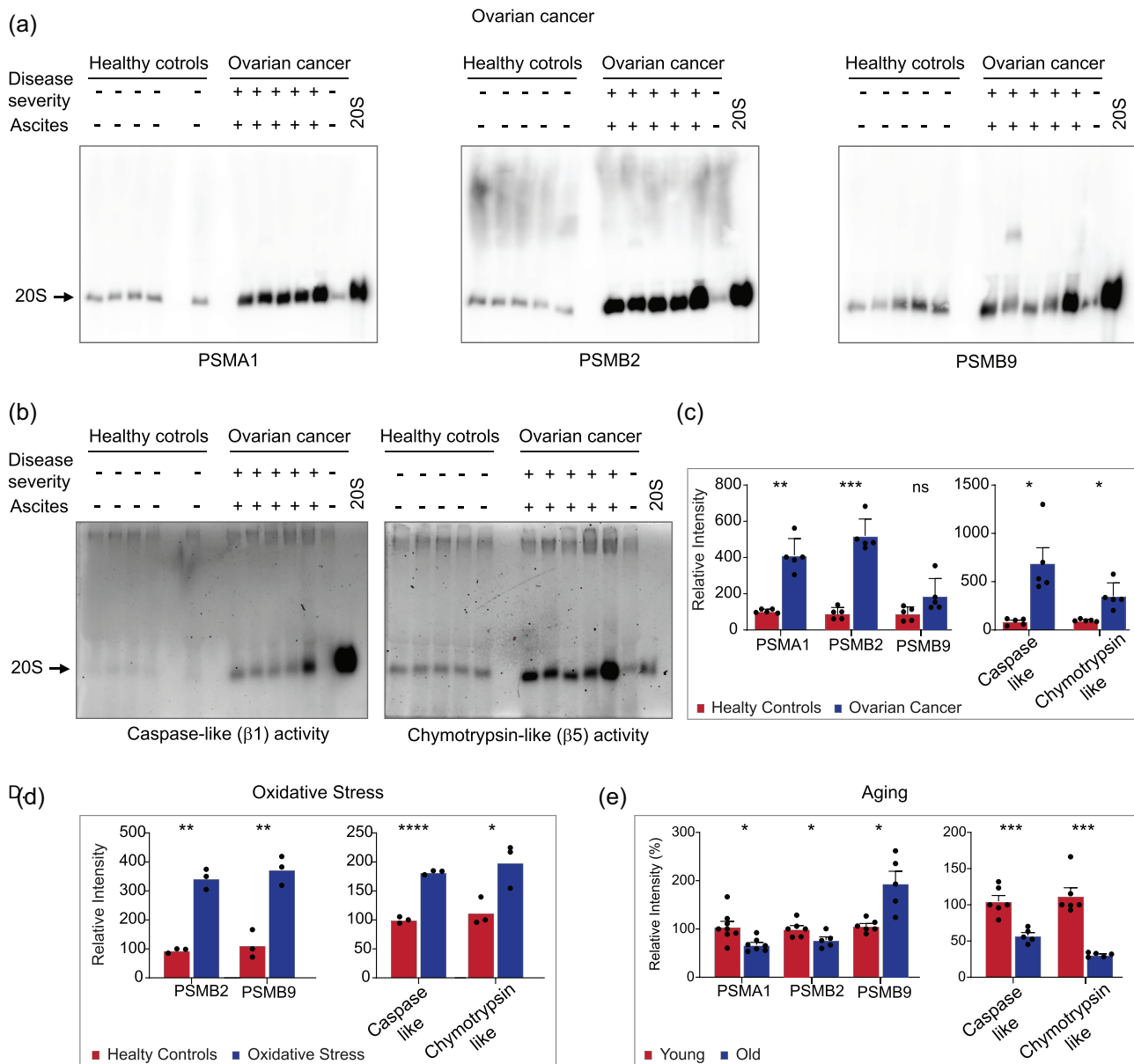
FIGURE 6 Physiological and pathological conditions influence the levels and activities of the c20S proteasome in blood. Proteasome levels were tested in three different conditions: ovarian cancer (a–c), oxidative stress (d), and aging (e). Native Western blots revealed that mice with ovarian cancer exhibited significantly higher amounts (a, c) and activities (b, c) of both constitutive and immunoproteasome c20S. (d) Plasma from 8-month-old mice with constitutive oxidative stress showed elevated levels of both constitutive and immunoproteasomes, along with increased peptidase activities. (e) Serum from 6 to 8 week old (young) mice and 1.2-year-old (old) mice, separated on native gels, indicated that aged mice exhibited a moderate reduction in c20S levels, while immunoproteasome levels nearly doubled with age. However, the peptidase activity of the c20S decreased with age. Bars represent averages, and errors represent SE. Values were compared by unpaired t-test with Welch's correction. Asterisks denote p-values of Caspase-like activity – 0.0178, Chymotrypin-like activity – 0.0149, PSMA1 - 0.0016, PSMB2 – 0.0002 for ovarian cancer (c), Caspase-like activity - <0.0001, Chymotrypin-like activity – 0.0382, PSMB2 – 0.0048, PSMB9 – 0.003 for oxidative stress (d), and Caspase-like activity – 0.0009, Chymotrypin-like activity – 0.0007, PSMA1 – 0.0103, PSMB2 – 0.0396, PSMB9 - 0.0227 for aging (e).

we found that the caspase- and chymotrypsin-like activities increased significantly, by approximately 7- and 3.5-fold, respectively (Figure 6b,c). Interestingly, one female mouse (first from the right in the group induced for ovarian cancer) showed no elevation in the levels and activities of the c20S proteasome. This mouse also did not develop detectable tumors upon postmortem examination and did not suffer from ascites, which are very common in the advanced stages of ovarian cancer (Iyer et al., 2021). This further links the levels and activity of the c20S proteasome with the progression of the cancer.

Oxidative stress is known to induce a rapid elevation in the catalytic and proteolytic capacity of 20S proteasomes, consistent with their role in eliminating oxidatively damaged proteins (Pickering & Davies, 2012). Moreover, prolonged exposure to oxidative stress leads to increased expression of 20S proteasome subunits (Wang et al., 2010). To investigate whether chronic oxidative

stress affects the levels and activity of blood proteasomes, we utilized a mouse line with a ceramide synthase 2 (CerS2) null mutation, resulting in a deficiency in synthesizing very long acyl chain ceramides (Zigdon et al., 2013). These mice suffer from various pathologies, including elevated levels of reactive oxygen species due to impaired complex IV activity in the mitochondria. We found that these mice exhibit elevated c20S proteasome levels and activities in the serum, compared to healthy control animals. A parallel increase in the levels of the immunoproteasome was also observed in the diseased animals (Figure 6d and Figure S13A,B), further establishing the connection between the 20S proteasome and oxidative stress.

Lastly, we examined the activity and levels of the c20S proteasome during aging. Multiple studies indicate that as organisms age, various proteasome-related deficiencies are observed, including a reduction in the chymotrypsin-like activity, changes in the relative levels of immunoproteasome subunits, downregulation of catalytic subunits and elevated oxidation-related modifications, which cumulatively reduce proteasome activity in cells (reviewed in Frankowska et al. (2022)). We compared the activity and levels of the c20S proteasome in the blood of young (6 to 8 week old) and old (1.2 years old) mice. Interestingly, we found that the levels of the constitutive c20S were moderately reduced (36% and 23% decline in the levels of PSMA1 and PSMB2, respectively), accompanied by activity reduction (∼50% decline in the caspase-like activity and over 70% decline in the chymotrypsin-like activity). The immunoproteasome, however, nearly doubled its levels in the serum during aging (Figure 6e and Figure S13C,D), suggesting that it responds to conditions involving chronic inflammation, oxidative stress, and compensatory mechanisms to maintain protein homeostasis and immune function in the face of age-related physiological changes.

In conclusion, our results demonstrate that similar to humans, the levels and activity of c20S proteasomes in mice are influenced by various physiological and pathological conditions. This suggests that mice are a valid model system for studying the c20S complex, providing a valuable tool for elucidating the mechanisms underlying proteasome regulation in health and disease.

4 | DISCUSSION

Until now, our knowledge of circulating proteasomes has mainly come from clinical studies (Dwivedi et al., 2021; Fukasawa et al., 2015; Hoffmann et al., 2011; Kakurina et al., 2017; Ma et al., 2009; Manasanch et al., 2017; Spektor & Berenson, 2017). The scientific community's primary focus on intracellular proteasomes and the limited attention to blood proteasomes were further constrained by the technical challenges associated with studying these tissue-dependent complexes (Hershko & Ciechanover, 1998). However, unraveling the molecular intricacies of circulating proteasomes is imperative, given their inherent presence in the blood and the wide spectrum of clinical conditions associated with elevated proteasome concentrations (Dwivedi et al., 2021; Fukasawa et al., 2015; Hoffmann et al., 2011; Kakurina et al., 2017; Ma et al., 2009; Manasanch et al., 2017; Spektor & Berenson, 2017). In this study, we used a transgenic mouse model with tagged proteasomes and employed MS-based and biochemical approaches to comprehensively investigate circulating proteasomes (Figure 7). Our findings revealed that the free uncapped 20S particle is the key circulating proteasome component in serum. Interestingly, we did not detect chaperones involved in proteasome biogenesis in the blood, suggesting that the complex is not assembled on-site but rather transported to it. Both constitutive and immuno c20S proteasomes are present in serum, displaying all three catalytic activities. Moreover, we identified cysteinylation and glutathionylation modifications on the c20S proteasome, setting it apart from its intracellular liver counterpart and likely influencing its catalytic properties. Specifically, we observed that compared to intracellular proteasomes, c20S exhibits enhanced caspase-like activity, while the chymotrypsin-like activity was reduced. Furthermore, we demonstrate that changes in physiological conditions lead to a significant increase in c20S proteasome levels in mice, mirroring observations in humans. Collectively, our results provide new insights into the biology of circulating proteasomes and highlight the specialization of blood 20S proteasomes.

The proteasome is a versatile complex capable of adopting multiple configurations. The 20S particle, for instance, can exist as a free proteasome or be capped with one or two of the activator particles: 19S, PA28αβ, PA28γ and PA200 regulators (Cascio, 2021; Pickering & Davies, 2012). These configurations are all found within cells, with their abundance varying based on the cellular context and environmental conditions. In serum, however, our observations indicate that the free 20S proteasome is the dominant configuration. We did not detect higher molecular weight forms of the 20S proteasome, such as the PA28αβ complex or the 19S proteasome, nor did we find associations with PA28γ or PA200 in mouse sera. Furthermore, our proteomic analysis of mouse serum did not identify any of these regulatory subunits. The nuclear localization of PA28γ and PA200 likely explains their absence in blood serum (Cascio, 2021; Ustrell et al., 2002; Yazgili et al., 2022). Interestingly, some proteomic analyses of human blood samples have reported subunits of the 19S and PA28αβ regulatory particles (Ben-Nissan et al., 2022). However, the levels of these subunits were found to be nearly 10 times lower than 20S proteasome subunits in a recent human serum profiling study, further supporting our observation that the uncapped 20S proteasome is the dominant form in serum (Dey et al., 2019). It is also important to note that the detection of 19S subunits does not necessarily imply their association with the 20S proteasome. Additionally, the 26S proteasome requires ATP to maintain the interaction between the 19S and 20S particles. However, the ATP concentration in plasma is approximately 1000 times lower than in the cellular environment (3 to 10 mM ATP within cells and 1 μM in plasma) (Chen & Xia, 2021; Gorman et al., 2007; Greiner & Glonek, 2021), hindering the stability of the complex. Moreover, unlike the 26S proteasome degradation pathway, which involves a complex enzymatic cascade with three different
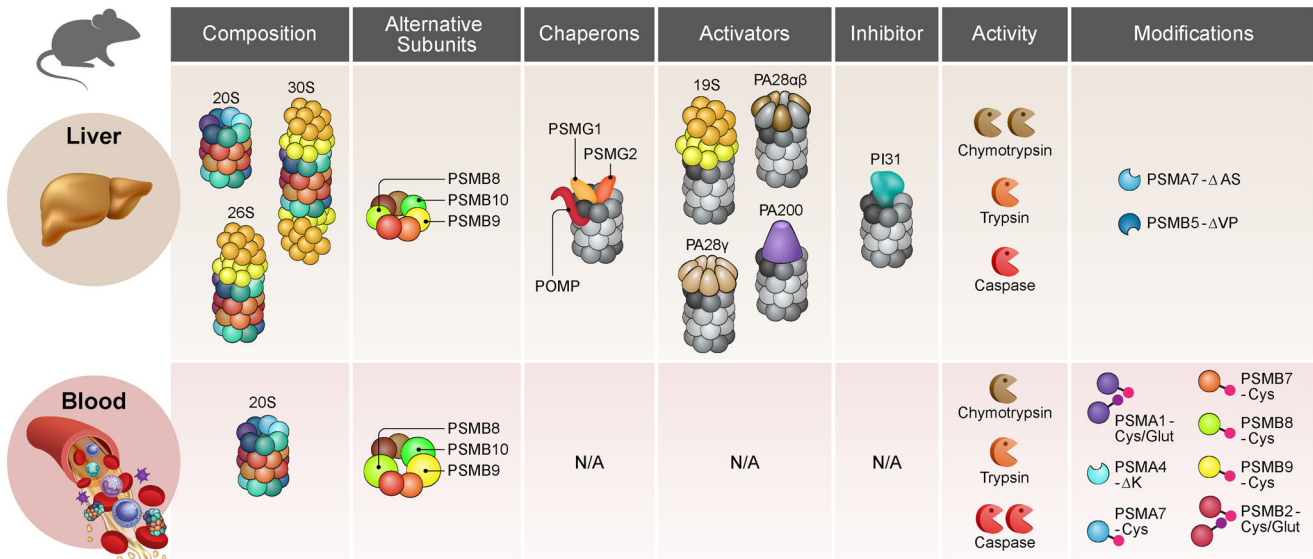
FIGURE 7 An illustrative summary of distinctive features of the circulating proteasome. In liver cells, various proteasome complexes (20S, 26S and 30S) are present, while in serum only the 20S particle is detected. Both tissues contain the immune proteasome subunits PSMB8, PSMB9 and PSMB10, with immunoproteasome subunits significantly enriched compared to their levels in liver proteasomes. However, chaperones involved in proteasome assembly (PSMG1, PSMG2 and POMP) were only found in liver immunoprecipitates, and not in serum, suggesting that proteasomes are assembled intracellularly and then transported into the bloodstream. Proteasome activators (PA28αβ, PA28γ and PA200) and the inhibitor PI31, which are present in liver, were also absent in serum. Both liver and serum proteasomes exhibit all three catalytic activities, but the serum proteasome shows enhanced caspase-like activity, whereas the liver proteasome has higher chymotrypsin-like activity. Additionally, proteasome subunits from liver and serum show different PTMs. In the liver, PSMA7 and PSMB5 subunits are truncated at their C-terminal residues, while in serum, cysteinylation, and/or glutathionylation are observed on PSMA1, PSMA7, PSMB2, PSMB7, PSMB8 and PSMB9 of the 20S proteasome.

types of enzymes (E1, E2 and E3) that ubiquitinate substrates in an ATP-dependent manner, the 20S proteasome does not require such a sophisticated process (Abi Habib et al., 2022). Therefore, the preference for 20S proteasome-mediated degradation in serum may arise from the simplicity and self-reliance of the system.

Another way to enhance the functional diversity and adaptability of the proteasome system is through the formation of specialised 20S proteasome subtypes (Abi Habib et al., 2022; Watanabe et al., 2022). These subtypes involve variations in the composition of the catalytic subunits, where the constitutive subunits PSMB5, PSMB6 and PSMB7 can be completely or partially replaced by immunoproteasome catalytic subunits PSMB8, PSMB9 and PSMB10. This process results in the generation of immunoproteasomes and two intermediate species. Our study revealed that c20S proteasomes encompass all six catalytic subunits, with immunoproteasome subunits significantly enriched compared to their levels in liver proteasome. Although we cannot yet determine whether all four forms of 20S proteasome subtypes—constitutive, immune and two intermediate proteasomes—coexist or their relative abundances, it is clear that the inclusion of immuno subunits within the c20S proteasome broadens the range of catalytic activities, influencing the repertoire of cleavage sites and peptide profiles (Abi Habib et al., 2022; Watanabe et al., 2022). Previously, it has been shown that PSMB8-containing 20S proteasomes (immuno and two intermediate proteasomes) are more efficient in degrading oxidized proteins, thereby shaping the 20S proteasome substratome (Abi Habib et al., 2022). Therefore, future studies identifying the population composition of c20S in blood under healthy and disease states are crucial for understanding the influence of various 20S proteasome subtypes on disease progression.

One of our interesting observations was the identification of cysteinylation and/or glutathionylation on PSMA1, PSMA7, PSMB2, PSMB7, PSMB8 and PSMB9. These covalent and reversible modifications involve the addition of glutathione (Xiong et al., 2011) or cysteine (Cysteinylation of Proteins, 2019) onto a cysteine residue. Comparative assessment indicates that the intracellular and extracellular cysteine levels are quite comparable (∼55 μM in livers (Gaitonde, 1967), ∼30 μM in whole blood (Chen et al., 1990) and ∼35 μM in plasma (Iyer et al., 2009; Watson et al., 2020)). However, intracellular glutathione levels are 1–2 orders of magnitude higher than the extracellular concentration (1-10 mM in cells (Vairetti et al., 2021) and ∼25 μM in plasma (Anderson & Meister, 1980; Iyer et al., 2009; Roman et al., 2013; Watson et al. (2020)). Despite this, neither cysteinylation nor glutathionylation, were found in any of the biological repeats of mouse liver proteasomes, suggesting that these modifications are not random but rather regulated.

The impact of S-glutathionylation on proteasome activity has been debated, with studies suggesting it can either activate or inhibit the complex's chymotrypsin-like activity and influence gate opening (Demasi et al., 2001; Demasi et al., 2003; Silva et al., 2012). Conversely, cysteinylation has been reported to suppress trypsin-like activity (Dick et al., 1992). In this study, we demonstrate that the c20S proteasome exhibits all three catalytic activities. However, when compared to the liver's cellular proteasome,

the c20S shows reduced chymotrypsin-like activity, while its caspase-like activity is notably enhanced. Interestingly, differential proteasome activity among these catalytic sites has been observed in various disease states, including cardiac hypertrophy (Drews et al., 2010), cancer (Deng et al., 2015; Shashova et al., 2017; Shashova, Kolegova et al., 2017) and ischemia (Sanchez et al., 2016). Here, we reveal that even within the same healthy organism, different tissues—specifically serum and liver—exhibit distinct catalytic activity profiles. The molecular details driving the enhanced caspase-like activity of the c20S proteasome and its implications on substrate degradation remain important questions for further exploration.

The 20S proteasome degrades proteins that contain fully or partially disordered regions that can enter into its narrow orifice (Ben-Nissan & Sharon, 2014; Kumar Deshmukh et al., 2019). This suggests that circulating serum proteins with intrinsically disordered regions, such as albumin (Litus et al., 2018), osteopontin (Dianzani et al., 2017), fibrinogen (Weisel & Litvinov, 2017) and serum amyloid A (Frame & Gursky, 2016), could serve as potential substrates for c20S, as demonstrated in our findings with galectin-3. Importantly, 20S proteasomes are capable of more than just complete degradation; they can also cleave proteins at specific sites, producing functional cleavage products (Olshina et al., 2018). This implies that c20S-mediated proteolysis may contribute to various physiological processes, including the activation of zymogen proteases (Nemerson & Furie, 1980), involvement in the coagulation and complement cascade (Wildes & Wells, 2010) and the generation of physiologically relevant peptide fragments of circulating proteins (Lechowicz et al., 2020). Additionally, the degradation of circulating proteins by c20S may act as a protective mechanism, clearing misfolded proteins that have lost their structure due to the highly oxidizing nature of serum compared to the intracellular environment (Alayash, 2022). Dendritic cells, present in the blood, specialize in cross-presentation of exogenous antigens on MHC class I molecules (Castell-Rodríguez et al., 2017; Embgenbroich & Burgdorf, 2018), and c20S proteasomes may contribute by generating antigenic peptides. In this context, immunoproteasomes, with distinct cleavage specificity from constitutive proteasomes (Goetzke et al., 2021), could generate diverse antigenic peptides. Overall, while the precise functional role of c20S proteasomes remains unclear, the presence of this active complex in the blood and its concentration dependency on disease states suggest it holds a physiologically relevant function. Future investigations will be crucial in elucidating this important question.

In summary, the gaps in our understanding of circulating serum proteasomes underscore the necessity to transition away from cell-line-based research, which has historically guided our insights into intracellular degradation. Instead, there is a call for investigations that more accurately capture the physiologically relevant context of extracellular proteasomes. Our observation that, in mice, conditions such as ovarian cancer, chronic oxidative stress, and aging induce an changes in c20S proteasome levels, mirroring findings in humans, positions mice as a valuable model for studying this extracellular complex in both physiological and pathological conditions. Overall, we believe that the findings presented here, spanning configuration, subunit composition, activity analyses, and establishing mice as a relevant model system for studying c20S proteasomes, will provide a foundation for addressing many remaining open questions. For instance, the origin of c20S proteasomes is still unclear, with uncertainty about whether they are derived from blood cells (e.g., erythrocytes, thrombocytes, monocytes, T cells), endothelial cells, or released via EV shedding. Furthermore, additional research is needed to explore the role of circulating proteasomes in health and disease, including how c20S proteasomes influence biological processes and whether their activity is linked to disease progression or immune responses.

AUTHOR CONTRIBUTIONS
Yegor Leushkin, David Morgenstern, and Gili Ben-Nissan performed the experiments. Shifra Ben-Dor designed the CRISPR approach. Rebecca Haffner-Krausz generated the CRISPR mice. Yegor Leushkin, Gili Ben-Nissan, David Morgenstern, Katharina Zittlau, and Michal Sharon analyzed the data. Yegor Leushkin and Gili Ben-Nissan prepared the figures. Michal Sharon, Yegor Leushkin and Gili Ben-Nissan wrote the manuscript.

CONFLICT OF INTEREST STATEMENT
The authors have no conflict of interest to declare.

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