The discovery of new functions for platelets, particularly in inflammation and immunity, has expanded the role of these anucleate cell fragments beyond their primary hemostatic function. Here, four in-depth human platelet proteomic data sets were generated to explore potential new functions for platelets based on their protein content and this led to the identification of 2559 high confidence proteins. During a more detailed analysis, consistently high expression of the proteasome was discovered, and the composition and function of this complex, whose role in platelets has not been thoroughly investigated, was examined. Biochemical analyses confirmed the presence of all catalytically active subunits of the standard 20S proteasome and immunoproteasome in human platelets, including β5, which was predominantly found in its precursor form. It was demonstrated that these components were assembled into the proteasome complex and that standard proteasome as well as immunoproteasome subunits were constitutively active in platelets. These findings suggest potential new roles for platelets in the immune system. For example, the immunoproteasome may be involved in major histocompatibility complex I (MHC I) peptide generation, as the MHC I machinery was also identified in our data sets. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.031757, 3308–3319, 2014.

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Although first described over a century ago, new roles and functions for platelets continue to emerge. Derived by budding from megakaryocytes and devoid of a nucleus, platelets were formerly not thought to produce proteins and their one role was to initiate and perform blood clotting. However, this view has changed in recent years; platelets have mRNA, microRNAs to regulate their mRNA, the machinery to synthesize proteins and they use it (1, 2). Furthermore, in addition to their function in hemostasis, it has been recognized that platelets play a role in inflammatory processes (3, 4). Through their interactions with the endothelium and other blood cells, platelets are believed to play a critical role in defense, wound repair, and more (5). Understanding of many of the new aspects of platelet function is still limited, but these recent advances raise the question of what other features are awaiting discovery that might be hidden in these small cell fragments.

There are limited methods available with which to study platelets; DNA-based methods cannot be applied, and although mRNA is present in platelets, its low level only allows for restricted analysis. Mass spectrometry (MS)-based proteomics is particularly well set up to study platelets, and previous studies have analyzed the platelet proteome (6–11), various subproteomes (12–16), and have shed light on aspects of platelet signaling and function (17–21). In this study, proteomic analysis of human platelets was conducted, generating an inventory of platelet proteins, which was then explored by comparison to proteomic data sets of nucleated cells with the aim of identifying new biology-related functions. This approach revealed consistently high expression of the proteasome, the protein complex that is the main protein degradation machinery in cells (Fig. 1). The presence of the proteasome in platelets has been described earlier (22). It is known to be active and its activity increases in response to agonist stimulation (23); however, a detailed analysis of the many subunits of this multimeric complex has not been performed and its role in platelets, which produce less protein than nucleated cells, is not fully understood. The proteasome’s core complex, the 20S proteasome, is composed of 28 nonidentical subunits, arranged in four rings, two compris-
The standard 20S core (middle) is composed of 28 nonidentical subunits that are arranged in four rings; two composed of seven α subunits and two composed of seven β subunits. Three of the β subunits (β1, β2, and β5) are catalytically active. The 19S regulator is composed of a base, containing six ATPase subunits and two non-ATPase subunits, and a lid, which contains up to ten non-ATPase subunits. The 20S proteasome and two 19S regulators form the 26S proteasome (left). The immunoproteasome, which is induced by IFNγ, contains three different catalytically active subunits (β1i, β2i, and β5i). The 11S regulator, which consists of heptameric complexes containing PA28α and β subunits, is also induced by IFNγ and can replace the 19S regulator (right).

Platelet opening up the possibility of new roles for these anucleate players, and further illustrates the critical role proteomics plays in improving our understanding of platelet function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hela, HEK293, and Jurkat cells (American Type Culture Collection (ATCC) Manassas, VA) were grown in Invitrogen Dulbecco’s modified medium (high glucose) containing L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin (both Invitrogen/Invitrogen, Burlington, ON). IFNγ was obtained from eBioscience (San Diego, CA). Protein concentrations were determined using a BCA assay (Thermo Scientific, Rockford, IL). Antibodies were from Boston Biochem (Cambridge, MA; mouse monoclonal anti-α1, clone 1A10–3G12), Enzo Life Sciences (Farmingdale, NY; mouse monoclonal anti-β5i, clone LMP7; rabbit polyclonal anti-β5; rabbit polyclonal anti-β3i; and mouse monoclonal anti-α2, clone MCP21), Santa Cruz Biotechnology (Santa Cruz, CA; mouse monoclonal anti-GAPDH), and Bio-Rad (Mississauga, ON; goat anti-mouse and anti-rabbit horseradish peroxidase). Activity probes MV151 and BioVSY were purchased from Leiden Institute of Chemistry (Leiden, The Netherlands). Enzymatic digestion for mass spectrometry analysis was performed using sequencing grade modified trypsin (Promega, Madison, WI).

**Ethics Statement, Blood Donations, and Platelet Preparation**—Ethical approval was granted by the University of British Columbia Research Ethics Board (certificate number H07–01943) and written informed consent was granted by the donors. Whole blood was drawn from the antecubital vein of healthy human volunteers into acid-citrate-dextrose at a final volume of 15%. Platelets were isolated by centrifugation, washed twice in buffer (10 mM trisodium citrate, 30 mM dextrose, and 10 units/ml aprotase), and resuspended in Tris-buffered saline/5 mM EDTA. During all steps of preparation, care was taken to avoid activation of platelets and isolated platelets were rested before experiments for half an hour at RT. Platelets, red blood cells (RBCs), and white blood cells (WBCs) were counted using an Advia 120 Automated Hematology Analyzer (Siemens Canada, Mississauga, ON).

**Fig. 1. Composition of the proteasome and immunoproteasome.** The standard 20S core (middle) is composed of 28 nonidentical subunits that are arranged in four rings; two composed of seven α subunits and two composed of seven β subunits. Three of the β subunits (β1, β2, and β5) are catalytically active. The 19S regulator is composed of a base, containing six ATPase subunits and two non-ATPase subunits, and a lid, which contains up to ten non-ATPase subunits. The 20S proteasome and two 19S regulators form the 26S proteasome (left). The immunoproteasome, which is induced by IFNγ, contains three different catalytically active subunits (β1i, β2i, and β5i). The 11S regulator, which consists of heptameric complexes containing PA28α and β subunits, is also induced by IFNγ and can replace the 19S regulator (right).
Immunoproteasome in Platelets

ON). These counts were used to estimate percentage RBC and WBC contamination. Although care was taken to minimize RBC and WBC contamination in all platelet preparations, for certain experiments extra precautions were taken, with platelets prepared using only the upper 1/3 of the platelet-rich plasma and visual exclusion of red blood cell contamination. Experiments were performed using platelets from three different donors unless otherwise indicated.

Global Proteomic Studies—Washed platelets from four individual platelet donors (PLT 1–4) as well as Jurkat and HEK293 cells were re-suspended in lysis buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TX-100, 2.5 mM Na pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1X protease inhibitor mixture (Calbiochem/Millipore, Billerica, MA), and immediately snap-frozen in liquid nitrogen. Lysates were mixed with nonreducing Laemmli buffer, boiled at 99 °C for 5 min and ∼250 μg protein/sample was separated by large (20 cm) 1-dimensional (1D) SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and each lane was cut into 40 bands. Standard tryptic in-gel digestions were performed overnight at 37 °C, without reduction or alkylation. Following digestion, peptides were extracted for MS analysis as described previously (25).

Liquid Chromatography Tandem Mass Spectrometry and Data Analysis—Separation and identification of peptides was performed by nano-HPLC MS/MS on an Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatography/mass spectrometry (LC/MS/MS) system. Separation and identification of peptides was performed by nano-HPLC MS/MS on an Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatography/mass spectrometry (LC/MS/MS) system. Three bands from 20–30 kDa were excised, reduced and alkylated, with Coomassie Brilliant Blue and each lane was cut into 40 bands. Standard tryptic in-gel digestions were performed overnight at 37 °C, without reduction or alkylation. Following digestion, peptides were extracted for MS analysis as described previously (25).

Identification of proteins was performed by extracting the Mascot generic format files from the MS data using DTA Super Charge version 2.0b1, part of the MSQuant open source project (http://msquant.sourceforge.net) and searching them against ENSEMBL human database (GRCh37.60, total proteins used: 74,741) using the X!Tandem algorithm TORNADO (2009.04.01.4) (26, 27) at the GPM database (http://gpmdb.thegpm.org/). The following search criteria were used: trypsin cleavage specificity with up to one missed cleavage site, no fixed modifications, variable modifications of oxidized methionine and dioxidized cysteine (sulfenic acid), and ± 0.2 ppm peptide tolerance and ± 0.4 Da MS/MS tolerance, ENSEMBL accession numbers, which are based on genomic information and therefore each protein isoform is assigned a different accession number, were used. A protein expect score (log(e) cut-off of −2 was chosen, which equates to a 1 in 100 chance of a stochastic protein assignment. The data sets are archived in the GPM database (http://gpmdb.thegpm.org/) and can be accessed by searching for their GPM numbers (supplemental Table S1). The overall quality of the data set was assessed by using the false positive rate (FPR), which is defined as: FPR (%) = 100 × [10^(-10^-e)]/N by the GPM, resulting in FPR values of 0.80% (PLT1), 0.40% (PLT2), 0.54% (PLT3), and 0.48% (PLT4), respectively.

Comparison to Published Human Platelet Proteome—To compare the differences and the overlap to a published human platelet proteomic data set (11), accession IDs were linked using our in-house database. Because each data set used different proteomic analysis strategies, different spectral matching algorithms, and a different protein sequence database, the following strategy was chosen. ENSP protein accessions (Current data set) were converted to Uniprot/Swiss Protein IDs (Identifier) and linked to the database used in the Burkhart et al. manuscript. This can result in a one-to-one, one-to-many, or many-to-one mapping. Alternatively, we selected Uniprot/Swiss Protein IDs (the database used in the Burkhart et al. manuscript) as the baseline and linked them via our database to Ensemble (ENSP protein accessions). This can also result in a one-to-one, one-to-many, or many-to-one mapping.

Western blot and Glycerol Gradient Analysis, Immunoprecipitation—Expression of the immunoproteasome in Hela cells was induced by incubation for 48 h with 150 units/ml IFNγ at 37 °C. For Western blot analysis, 50 μg of platelet or Hela cell lysate were separated on a 12% SDS-PAGE.

For glyceral gradient analysis, platelets were lysed by three freeze/thaw cycles (liquid nitrogen/30 °C). Four milligrams of protein lysate was separated on a 10–40% glycerol gradient (16 h, 4 °C, 25,000 rpm, SW41 rotor, Beckman Coulter, Mississauga, ON) and 18 fractions of 600 μl were taken from the top to the bottom and analyzed by Western blot. Standards (BSA, apoferritin, and 20S proteasome) were separated using a comparable gradient during the same run.

Immunoprecipitation of the 20S proteasome was performed mainly as described (28). Briefly, platelets were lysed by three freeze/thaw cycles (liquid nitrogen/30 °C) and 25 strokes in a Dounce homogenizer. Debris was removed by several centrifugation steps and 2 mg of the cytoplasmic fraction was incubated with Protein G Agarose (Thermo Scientific) for 2 h at 4 °C. The supernatant was incubated with 20 μg MCP21 or no antibody as control and 100 μl Protein G Agarose was added for the precipitation, which was performed overnight at 4 °C. Beads were washed three times, boiled with Laemmli buffer, and supernantant was separated on an “Any kDa” gel (Bio-Rad). Three bands from 20–30 kDa were excised, reduced and alkylated, and in-gel digested with trypsin. Peptides were analyzed by LTQ-Orbitrap and proteins were identified using the GPM and Mascot (29). For this, Mascot generic format files were extracted from the MS data using DTA Super Charge version 2.0b1. The X!Tandem algorithm CYCLOCNE (2010.12.01.2) (26, 27) in the GPM was applied to search against ENSEMBL human database (GRCh37.60, total proteins used: 82,080) using the following criteria: trypsin cleavage specificity with up to one missed cleavage site, fixed modification carbamidomethyl, variable modification of oxidized methionine, ± 20 ppm peptide tolerance, and ± 0.4 Da MS/MS tolerance. Protein score cutoffs were set to log(e) < −2 (GPM) and identification of at least two peptides for each protein were required. Data sets can be accessed by their GPM numbers (supplemental Table S2). Mascot version 2.3.01 and database SwissProt_57.1 (513,877 sequences) were used to identify proteins with the following criteria: trypsin cleavage specificity with up to one missed cleavage site, fixed modification carbamidomethyl, variable modification of oxidized methionine, ± 20 ppm peptide tolerance, and ± 0.6 Da MS/MS tolerance, and the scoring scheme was ESI-TRAP. Data sets are found in the supplemental data (IP1_F038211, IP2_F038244, IP3_F040058, and IP4Control_F038242), their according F-numbers are also listed in supplemental Table S2. A protein score of > 40 and identification of at least two peptides for each protein were set for further analysis and spectral counting was performed using emPAI, which is included in the Mascot search engine.

Proteasome Activity Assays—Proteasome activity probe assays were performed as described (30). Platelets were re-suspended to 4 × 10^6 cells/ml in Tris buffered saline, incubated with 1 μM MV151 or dimethyl sulphoxide as vehicle control for 1 h at 37 °C, and lysed in 1% Triton X-100 lysis buffer. Alternatively, cells were lysed in a buffer containing 0.1% Nonidet P-40 and 20 μg Hela or 50 μg platelet lysate was incubated with 10 μM MV151 or vehicle control for 1 h at 37 °C. To destroy proteasome activity, lysates were boiled with 1% SDS at 99 °C for 5 min before incubation with MV151. Samples were incubated with Laemmli buffer, separated on a 12% SDS-PAGE, and the gel was scanned using the TAMRA settings on a Typhoon 9400 (GE Healthcare).

Pull-down of active proteasome subunits was performed following the manufacturer’s protocol (Leiden Institute of Chemistry). Briefly, 1.5 mg platelet lysate was incubated with 15 μM BioVS for 1 h at 37 °C and after denaturation and reduction, and alkylation of the proteins,
the pull-down was performed by adding 50 μl Myone Streptavidin T1 Dynabeads (Invitrogen, Burlington, ON) for 1 h at RT. Proteins were eluted with Laemmli buffer and 10 μM biotin and separated on a 12% SDS-PAGE. Silver staining was performed and bands from 20–30 kDa were excised. Proteins were in-gel digested with trypsin, peptides analyzed by LC-MS/MS using a QStar XL Q-TOF (Applied Biosystems/Invitrogen, Burlington, ON), and proteins identified using the GPM and Mascot. Same databases and search criteria were applied as listed in the previous paragraph besides deviating peptide tolerances: 100 ppm (GPM) and 0.15 Da (Mascot). GPM and Mascot F-numbers are listed in supplemental Table S3 and Mascot data are found in the supplemental Data (PD1_1_F038123, PD1_2_F038126, PD1_3_F038129, PD2_1_F038670, PD2_2_F038671, PD2_3_F038672, and PD3_F039251).

RESULTS

Global Proteomic Analysis of Platelets—Global proteomic experiments were performed using four separate platelet preparations as well as two human cell lines, and proteins were identified using the GPM, where the generated data sets are archived (supplemental Table S1). The platelet (PLT) data sets (supplemental Table S4) contained 1818, 1279, 1501, and 1088 high confidence proteins (log(e) score <−2 with each protein having at least one unique peptide) following removal of common contaminants often found in MS experiments (e.g., trypsin and keratin). Combining these experiments resulted in the identification of 2559 platelet proteins, with 618 proteins being detected in all four data sets (Fig. 2A). Throughout the study, platelets were prepared using methods designed to minimize contamination from other blood cells. Percentages of WBC and RBC contamination were determined using an ADVIA 120 hematology analyzer at the end of the platelet preparation and were found to be at the lower limit of detection of the instrument, where the deviation range is higher than the number of counted cells: for WBCs, 0.01–0.06 × 10^9 cells/L were detected, but the deviation in this area can be up to 0.46 × 10^9 cells/L. RBCs were detected to be around 0.03–0.05 × 10^12 cells/L with the deviation being 0.03 × 10^12 cells/L (ADVIA 120 Hematology System Operator’s guide, Siemens, V2.02.00 2002–07, 2002). As it was difficult to draw a conclusion regarding contaminations because of these measurements, assessment of the purity of the samples was also conducted post-MS analysis using the plasma and RBC databases at the Normal Clinical Tissue Alliance (http://wiki.thegpm.org/wiki/Normal_Clinical_Tissue_Alliance). The top 30 (by count) proteins in both the plasma and RBC data sets were searched against the platelet data sets. The majority of the proteins were not found in the platelet data sets, and those that were found were ranked low (supplemental Tables SS and S6). Furthermore, the data sets were searched for the presence of CD45 and MHC II chains, proteins that are not found in platelets but are expressed in leukocytes. CD45 was detected in one data set (PLT3, rank 947, log(e) = −19.4) at a low rank indicating minor WBC contamination. An MHC II chain was found in a different data set (PLT4, rank 1088, log(e) = −2.2), but with very low confidence. Therefore, contamination from other blood components in the platelet data sets was concluded to be minimal.

To gain insights into platelet biology, comparative analysis of the platelet data sets and the data sets derived from the nucleated HEK293 and Jurkat cell lines that had been obtained using the same protocols and instruments was performed using data mining tools at the GPM, including analysis of protein gene ontology and pathway analysis using Kyoto Encyclopedia of Genes and Genomes (31–33). Several features clearly related to the anucleate nature of platelets were evident in comparison to the cell lines, including very low numbers of proteins associated with the ribosome, the spliceosome, translation and transcription, and a significant de-
plation of nuclear proteins and proteins associated with the cell cycle. Additionally, categories relating to hematopoietic cell lineage and the coagulation and complement cascades are over-represented in platelets when compared with the two nucleated cell lines. Comparative analysis was also conducted between our platelet proteomic data set and the data set generated by Burkhart et al. (11) that was published during the preparation of this report. Their study applied a multitude of approaches leading to the identification of ~4000 unique proteins. As both analyses used different databases and a different strategy, their resulting protein lists were compared using two different approaches, described in the Experimental Procedures. This led to the identification of 1761 versus 1735 proteins in common between the data sets, 2354 versus 2380 proteins unique for the Burkhart et al. data set and 716 versus 742 proteins only found in our data set (Fig. 2B and C; supplemental Tables S7 and S8).

The Proteasome and Immunoproteasome in Platelets: Proteomic and Western blot Analysis—The proteasome pathway was equally represented in data sets from both platelets and nucleated cells, a surprising finding as the proteasome is known to be found in the nucleus (PA28 regulator of the immunoproteasome. However, two proteins of the 19S proteasome, and PA28, which comprises the 11S regulatory subunit, were found in at least one data set, including all components of the 20S core, as well as the three immunoproteasome subunits, and both PA28 subunits were detected, whereas control experiments performed without a specific antibody identified only two subunits at a very low score (Table II). This further indicates that all of these subunits are incorporated into the 20S proteasome complex in platelets.

Activity of Proteasome Subunits in Platelets—Proteasome activity probes were used to determine which proteasome subunits in platelets are functional. Hela cells were again used as positive controls, whereas heat-inactivated samples served as negative controls. The fluorescence-labeled probe MV151 labeled the same proteasome subunits in platelets and platelet lysates as in IFN-γ-stimulated Hela cell lysates, indicating that the immunoproteasome as well as the standard proteasome are active in platelets (Fig. 5). To exclude the possibility that WBC contamination might be responsible for the detection of active immunoproteasome subunits, platelets were prepared with extra care (see Experimental Procedures) to eliminate any WBC contamination and assayed for proteasome activity using the MV151 probe. Identical results from these and “normal” preparations were obtained, indicating that the activity is indeed from platelets (supplemental Fig. S3).

Molecular & Cellular Proteomics 13.12

All 20S Proteasome Subunits in Platelets are Incorporated into the Complex—Glycerol gradient analysis was conducted to determine whether the proteasome subunits detected by Western blot analysis in platelets are incorporated into the proteasome complex (Fig. 4). The proteasome subunits α7, β5, β1i, and β5i were all detected mainly in one common fraction, in agreement with experiments performed in parallel using purified 20S protein as well as Hela cell lysate (supplemental Fig. S1). This indicated that standard as well as immunoproteasome subunits were incorporated in the proteasome complex. However, although some mature β5 was identified in the same fraction as the other subunits, the majority of β5, in the mature as well as in a higher molecular weight form, was detected in fractions 1–2 of the platelet preparations, whereas all β5 was detected in later fractions in Hela cells. This indicates that the majority of β5 may not be incorporated into the proteasome complex in platelets. To analyze all subunits of the 20S proteasome, immunoprecipitation using an antibody against the α2 subunit and subsequent LC-MS/MS analysis was performed. All α and β subunits of the 20S core, as well as the three immunoproteasome subunits and both PA28 subunits were detected, whereas control experiments performed without a specific antibody identified only two subunits at a very low score (Table II). This further indicates that all of these subunits are incorporated into the 20S proteasome complex in platelets.

MHC I Peptide Presentation Machinery is Present at Protein Level in Platelets—The immunoproteasome is believed to be the main proteasome isoform involved in MHC I peptide pres-
entation (24). Therefore, the original global proteomic data sets were searched for proteins involved in the MHC peptide presentation machinery and products from HLA genes (Table IV). All components of the machinery except for β2 microglobulin and BiP were identified. HLA A and B gene products were found in all data sets, and products for HLA C, E, and G were found in some, demonstrating that platelets have all the necessary machinery to be capable of MHC I peptide generation.

| Gene name | Protein subunit | PLT1 | PLT2 | PLT3 | PLT4 |
|-----------|----------------|------|------|------|------|
| Rpt1      | PSMC2          | -71.3| -10  | -13  | -12.3|
| Rpt2      | PSMC1          | -84.3| n.d. | -70.3| -16.9|
| Rpt3      | PSMC4          | -26.5| -54  | -62.5| -27.4|
| Rpt4      | PSMC6          | -76.5| -8.4 | -54.1| -7   |
| Rpt5      | PSMC3          | -81.5| -8.6 | -143.1| -46.8|
| Rpt6      | PSMC5          | -70.7| -28.3| -58.1| -33.3|
| Rpn1      | PSMD2          | -20.6| -26.9| -78.2| -65.4|
| Rpn2      | PSMD1          | -14.6| -58  | -50.1| -19.3|
| Rpn3      | PSMD3          | -116.2| -48.4| -127.8| -34.3|
| Rpn5      | PSMD12         | -11.8| -17  | -64.3| -27.9|
| Rpn6      | PSMD11         | -111.3| -14.4| -56.2| -88.3|
| Rpn7      | PSMD6          | -10.1| n.d. | -10.4| n.d. |
| Rpn8      | PSMD7          | -19.7| -25.1| -33.3| -8.3 |
| Rpn9      | PSMD13         | -26.1| -16.7| -42.8| -12.1|
| Rpn10     | PSMD4          | -49.5| n.d. | -27.9| -28.2|
| Rpn11     | PSMD14         | -12.2| -33.4| -44.5| -2.1 |
| Rpn12     | PSMD8          | -2.74| -12.2| -22.3| -4   |
| Rpn4      | PSMD9a         | -26.5| n.d. | -11.2| -2.3 |
|           | PSMD5a         | -17.2| n.d. | -6.9 | -2.9 |
|           | PSMD10a        | -13  | n.d. | -11  | -3.1 |

| Gene name | Protein subunit | PLT1 | PLT2 | PLT3 | PLT4 |
|-----------|----------------|------|------|------|------|
| α6        | PSM1           | -104.6| -11.8| -4   | -62.7|
| α2        | PSM2           | -92.1| -64.2| -35.9| -22.1|
| α7        | PSM3           | -46.6| -27.1| -41  | -14.3|
| α3        | PSM4           | -53.3| -24  | -43  | -48.1|
| α5        | PSM5           | -81.9| -14.4| -25.8| -30.2|
| α1        | PSM6           | -45.8| -22.3| -36.7| -26.9|
| α4        | PSM7           | -75.5| -61.4| -6.9 | -24.3|
| β6        | PSMB1          | -55.8| -7.5 | -51.2| -35.6|
| β4        | PSMB2          | -77.8| n.d. | n.d. | n.d. |
| β3        | PSMB3          | -55.3| -32.1| -33  | -18.4|
| β7        | PSMB4          | -47.9| -7.5 | n.d. | -28.6|
| β5        | PSMB5          | (1.4)| n.d. | n.d. | n.d. |
| β1        | PSMB6          | -34  | n.d. | -5.3 | (1-1.1) |
| β2        | PSMB7          | -9.7 | n.d. | -4.5 | n.d. |
| β5i       | PSMB8          | -34.1| -4.8 | -3.3 | -11.6|
| β1i       | PSMB9          | -43.9| n.d. | n.d. | n.d. |
| β2i       | PSMB10         | n.d. | n.d. | n.d. | n.d. |
| β5t       | PSMB11         | n.d. | n.d. | n.d. | n.d. |

| Gene name | Protein subunit | PLT1 | PLT2 | PLT3 | PLT4 |
|-----------|----------------|------|------|------|------|
| PA28α     | PSEM1          | -146.5| -60.1| -129.8| -77  |
| PA28β     | PSEM2          | -51.6| -35.1| -87.3| -24.3|
| PA28γ     | PSEM3          | n.d. | n.d. | n.d. | n.d. |
| PA200     | PSEM4          | n.d. | n.d. | n.d. | n.d. |
| POMP      | PSMF1          | -3.2 | n.d. | n.d. | n.d. |

Chaperoneα, released after assembly of proteasome, nuclear proteinβ. n.d.: not detected.

**TABLE I**

Detection of proteasome subunits in the global proteome platelet datasets. The log (e) value from the GPM is listed for every subunit detected. Log (e) values below the cut-off threshold (≥ -2) are listed in brackets, and were not used for the global analysis.

**DISCUSSION**

Because of their small size and lack of correlation between RNA expression and protein abundance (11) that hampers microscopic and genomic approaches, proteomics has emerged as a key technology to study platelets. Here, we applied a global proteomic approach to gain new perspectives on the biological functions of platelets. This led to the identification of 2559 high confidence proteins, which we
have archived in a public database with the hope that this inventory will be of use to others in their efforts to improve understanding of platelet biology.

During the preparation of this manuscript, a more in depth data set was published by Burkhart et al. (11). Although this data set was used for additional comparative analysis, the two studies were performed using different databases so a direct comparison proved difficult, and mandated translation of one of the two data sets prior to computational matching. Common between both data sets, 1761 or 1735 proteins were found; their detection in two independent studies adds confidence that these proteins are truly present in platelets. This conclusion is corroborated by their relative abundance, which generally followed similar trends. As different proxies to estimate protein abundance were used (NSAF versus log(e)/log(I)), this observation validates the estimated protein abundances in Burkhart et al. In turn, this substantiates their conclusion of protein abundance in platelets not being accessible by transcriptomics, and demonstrates the importance of proteomics in contemporary platelet analysis.

Dependent upon the matching strategy we used, there were 716 or 742 proteins found only in our data set. Although a fraction of these are proteins not found in the Burkhart et al. data set, the majority arises from alternative forms of shared

![Fig. 3](image-url)

**Fig. 3.** Detection of four distinct proteasome and immunoproteasome subunits in human platelets by Western blot analysis. Hela cells, which synthesize immunoproteasome subunits only after stimulation with IFNγ, were used as controls. Subunit α7 was detected in platelets as expected and was used to show the general expression level of the proteasome. β5, which was not found in the global platelet proteome data sets, was detected by Western blot analysis, although the molecular weight of the main form of β5 in platelets was higher than in Hela cells, indicating that the precursor or a post-translationally modified version of β5 is predominantly present in platelets. The presence of the immunoproteasome subunits β5i and β1i was also confirmed by Western blot analysis. Detection of GAPDH was used to control for loading. Western blots are representative of at least three separate experiments.

![Fig. 4](image-url)

**Fig. 4.** Glycerol gradient analysis demonstrates incorporation of distinct proteasome and immunoproteasome subunits in the platelet proteasome complex. Platelet lysate was separated using a 10–40% glycerol gradient and 18 fractions were collected from the top to the bottom. Fractions 1–13 (10–30%) were analyzed using Western blot analysis for the presence of α7, β5, β1i, and β5i. All proteasome subunits were detected in fraction 7 indicating that they are all incorporated in the proteasome complex. However, the majority of β5, including the precursor was detected in fractions 1–2. Standard proteins (BSA-4.2S, apoferritin-17.6S, 20S proteasome) were separated on a glycerol gradient during the same run. Western blots are representative of the results of three separate glycerol gradients experiments performed using three independent blood donors.
proteins, and is explained by the GPMDB’s use of ENSEMBL database in our analysis, which unlike Uniprot matches proteins at the genomic level via ENSP entries. Conversely, the 2354 or 2380 additional proteins found by Burkhart et al. originate in their more comprehensive approach: they did not only use global analysis, but also TiO2 and membrane enrichment. A general trend of abundant proteins being present in both studies, and differences being more frequent for lower copy number proteins and proteins identified following enrichment could be observed. The gain in sensitivity because of iTRAQ labeling of less abundant peptides and subsequent pooling may also have contributed to the higher overall coverage in Burkhart et al.

During our analysis, the proteasome emerged as an interesting feature of anucleate platelets that warranted further investigation. This discovery was not achieved by a mere gene ontology analysis of the platelet data sets but by comparison with data sets of nucleated cells, which had been prepared using the same procedure and therefore could act as perfect control data sets. This highlights the significance of such controls when interrogating difficult-to-study biological systems such as platelets for unusual features. Using a combination of global proteomic analyses and traditional biochemical techniques we demonstrate that human platelets possess all components of the standard 26S proteasome as well as the immunoproteasome and that both forms of the proteasome are active. This adds to the mounting evidence that despite their limited protein synthesis, platelets have an active proteasome/ubiquitylation pathway (34–36).

The detection of immunoproteasome subunits in our initial proteomic study was surprising and changes our understanding of the 20S proteasome in platelets (22, 23, 37, 38). Activity-based probes were used to demonstrate that immunoproteasome as well as standard proteasome subunits were not simply present but contribute to the proteasome activity in platelets. The activity probes used bind specifically and irreversibly to catalytically-active proteasome β subunits and can therefore analyze the activity of single subunits (39) as demonstrated previously by studies performed on e.g. the thymoproteasome (30) and the proteasome in plants (40). It is worth considering that the results of earlier studies describing activity of the proteasome in platelets could, in part, be accounted for by the presence of active immunoproteasome subunits, and these studies could be re-evaluated in light of these new findings. Importantly, the expression and activity of the immunoproteasome in platelets did not require activation following IFNγ or other reagents and was consistent over a number of healthy donors.

We show that both forms of the proteasome are indeed found in platelets and are not derived from contaminating blood cells, something that is always a concern when studying platelets. Throughout the study, every effort was made to minimize contamination from other blood cells. In terms of these other blood cells, RBCs would not be a potential source

| Gene name | Proteasome subunit | IP1 GPM log (e) | Mascot Protein score | emPAI | IP2 GPM log (e) | Mascot Protein score | emPAI | IP3 GPM log (e) | Mascot Protein score | emPAI | Control GPM log (e) | Mascot Protein score | emPAI |
|-----------|--------------------|----------------|---------------------|-------|----------------|---------------------|-------|----------------|---------------------|-------|----------------|---------------------|-------|
| PSMA1 α6  | −64.2              | 131            | 1.33                |       | −75.9          | 111                | 0.7               | −63.5          | 64                  | 0.37              |       |
| PSMA2 α2  | −70.5              | 200            | 1.63                |       | −80.9          | 144                | 1.33              | −50.6          | 74                  | 0.83              |       |
| PSMA3 α7  | −47.5              | 119            | 0.25                |       | −42.6          | 113                | 0.39              | −46.5          | 113                 | 0.39              |       |
| PSMA4 α3  | −47.1              | 102            | 0.7                 |       | −43.2          | 72                 | 0.53              | −39.1          | 49                  | 0.53              | −2.3    |
| PSMA5 α5  | −33.3              | 111            | 0.43                |       | −16.8          | 108                | 0.81              | −27.1          | 80                  | 0.61              |       |
| PSMA6 α1  | −73.3              | 313            | 2.88                |       | −56            | 190                | 0.97              | −9            | 45                  | 0.25              | (−1.3) |
| PSMA7 α4  | −69                | 255            | 1.45                |       | −49.6          | 126                | 0.75              | −46.2          | 256                 | 0.96              |       |
| PSMB1 β6  | −60                | 165            | 0.8                 |       | −73.3          | 136                | 1.02              | −33.5          | 73                  | 1.02              |       |
| PSMB2 β4  | −37.9              | 50             | 0.31                |       | −53.3          | 109                | 0.97              | −22.9          | 50                  | 0.31              | −2.3    |
| PSMB3 β3  | −19.2              | 72             | 1.57                |       | −42.2          | 105                | 0.96              | −32.6          | 122                 | 0.73              |       |
| PSMB4 β7  | −37.6              | 224            | 1.19                |       | −60.1          | 218                | 0.54              | −49            | 117                 | 0.72              |       |
| PSMB5 β5  | −24.4              | 40             | 0.25                |       | −28.6          | 67                 | 0.73              | −7.2           | n.d.                 | n.d.              |       |
| PSMB6 β1  | −23.3              | 34             | 0.13                |       | −31.1          | 86                 | 0.45              | −9            | 57                  | 0.28              | −9.4    |
| PSMB7 β2  | −32.8              | 89             | 0.37                | n.d.  | n.d.          | n.d.                |       | −18.2          | 79                  | 0.23              |       |
| PSMB8 βi  | −36.1              | 116            | 0.67                |       | −32.3          | 65                 | 0.51              | −15.5          | 56                  | 0.36              | n.d.    |
| PSMB9 βi  | −19.6              | 95             | 0.5                 |       | −13.2          | 103                | 0.31              | −10.1          | 51                  | 0.14              |       |
| PSMB10 β2i| −17                | 99             | 0.24                |       | −29.2          | 190                | 0.38              | −9.1           | 86                  | 0.11              |       |
| PSME1 PA28α| −43.2              | 124            | 0.73                |       | −20.6          | 73                 | 0.12              | −2.1           | 27                  | 0.55              |       |
| PSME2 PA28β| −22.9              | 125            | 0.99                |       | −27.1          | 89                 | 0.58              | (−1.4)         | (19)                 | 0.12              |       |

**Proteasome subunits identified in human platelets following precipitation with the antibody MCP21 (anti α2). Three independent immunoprecipitation (IP) experiments using the cytoplasmic fraction of human platelets were performed and analyzed by LC-MS/MS. Proteins were identified using the GPM and Mascot (accession numbers can be found in Table S2) and log (e) values (GPM) and score and emPAI values (Mascot) for quantitative estimations are listed. Values below the cut-off threshold (GPM: log (e) > −2, Mascot: Protein score < 40) are listed in brackets. Proteasome subunits were not detected with similar scores in a control pull-down experiment performed without the antibody. n.d.: not detected.**
show the same pattern as the IFN
of the probe was detected using a fluorescence scanner. Platelets inactivated. Lysates were separated by SDS-PAGE and incorporation lysates and platelets were treated with DMSO or platelets were heat-sates or platelets were incubated with MV151. As controls, cell some subunits in platelets using the activity probe MV151.

![Image]

**Fig. 5. Identification of active proteasome and immunoproteasome subunits in platelets using the activity probe MV151.** Lysates or platelets were incubated with MV151. As controls, cell lysates and platelets were treated with DMSO or platelets were heat-inactivated. Lysates were separated by SDS-PAGE and incorporation of the probe was detected using a fluorescence scanner. Platelets show the same pattern as the IFN-γ-stimulated Hela cells demonstrating that proteasome as well as immunoproteasome subunits are active in platelets. The gel was later Coomassie Blue stained to demonstrate equal loading of comparable samples. Results are representative of three separate experiments.

of contaminating proteins, as they themselves do not have an immunoproteasome (41). Regarding WBCs, our original global proteomic experiments were performed using very clean platelet preparations, with CD45 and MHC II detected only at very low rank. Also, proteasome subunits located in the nucleus were not found in our data sets, as would be expected if the detected proteasomes had come from nucleated cells. Furthermore, the generation of an extra-pure platelet preparation did not lead to the loss of any active proteasome subunits, which again would be expected if the immunoproteasome activity was caused by WBC contamination. Also, the more in-depth analysis by Burkhart et al. showed evidence of proteasome as well as immunoproteasome subunits, supporting our notion of finding these proteins in platelets.

The discovery of an active immunoproteasome in platelets leads to further questions. First, where does it come from? As platelets only have limited protein synthesis, megakaryocytes may already possess the immunoproteasome; however, it cannot be excluded that immunoproteasome subunits are produced in the later stages of a platelet’s life. Then, which forms of the proteasome are actually present in platelets: the immunoproteasome, the standard proteasome, or mixed forms? As β5 was consistently found in very low levels com-
pared with all other subunits in platelets, mixed forms containing β5i, β2, and β1, which are known to exist (42, 43), might be present in platelets. It could also be that different forms of the proteasome are present in different platelet populations and/or in different subcellular compartments or they may coexist. As demonstrated in this study, IFN-γ stimulation is not necessary to generate the immunoproteasome in platelets. However, do other stimuli alter the proteasome composition or the activity? This might be indicated by recent studies that showed that platelet activation leads to increased proteasome activity (23) and that some proteasome subunits were found to be differentially regulated in the platelets of patients with coronary ischemic disease and acute coronary syndromes (44, 45).

Finally, what is the function of the platelet immunoproteasome? Besides degradation of redundant proteins, the proteasome and especially the immunoproteasome are involved in the generation of MHC I peptides (24). Our global proteomic analysis identified all key players of the MHC I pathway besides β2 microglobulin, which may have been lost during platelet preparation. MHC I chains and β2 microglobulin have been detected earlier by Western blot analysis in human platelets and by flow cytometry in murine platelets (46–48), but it is still a matter of debate whether these complexes are integrated in the membrane or are absorbed from plasma. The detection of the whole MHC I machinery in our global proteomic data set strongly indicates that MHC I complexes are generated and assembled by platelets themselves. Considering that platelets require only low proteasome activity to degrade proteins because of their limited production of proteins, we propose that the generation of MHC I peptides is even the major role of the proteasome in platelets, explaining the presence of the immunoproteasome, which is optimized for the generation of MHC I peptides (24). As platelets are the first cells encountered by pathogens entering through vascular injuries, presenting antigens derived from these intruders might be an important function of these blood components. Taking recent results into account, which have shown that exogenous peptides obtained through endocytosis can be cross-presented by MHC I (49) and that platelets are able to phagocytose (50), might platelets even be able to cross-present MHC I peptides? This possible role in the activation of the immune response is in agreement with the recent findings that the function of platelets is not restricted to hemostasis, but reaches beyond this to inflammation processes and more (3, 4, 51, 52).

Additionally, what does the presence of an active immunoproteasome in platelets mean therapeutically? Recognizing that platelets contain an active immunoproteasome, as well as gaining a clear understanding of the role of the proteasome/immunoproteasome in platelet function and viability, is crucial as both forms are targeted by chemotherapeutic agents (53). However, proteasome inhibitors have been shown to inhibit platelet aggregation (54), and a recent study demonstrated a negative
effect of proteasome inhibitors on platelet life span, through proteasomal regulation of pro- and anti-apoptotic proteins (55). More recently immunoproteasome-specific inhibitors have been investigated as anti-cancer agents, with the hope that these may be more specific and have less side effects than proteasome inhibitors. Initial studies are very promising (53, 56); however, in light of our discovery of an active immunoproteasome in platelets, it should be considered that these newer reagents could have the same negative effect on platelet biology as regular proteasome inhibitors.

Further insights into the regulation of the platelet proteasome/immunoproteasome in vivo would require clinical studies and/or animal experiments, which are beyond the scope of this study, but we expect that our findings provide impetus for other groups to further explore the role of the proteasome in platelets. Finally, this report illustrates that additional platelet

### Table III

| Gene name | Protein Ensemble number | Peptides identified | Protein score | Peptides identified | Protein score | Peptides identified | Protein score | Peptides identified | Protein score |
|-----------|------------------------|--------------------|--------------|--------------------|--------------|--------------------|--------------|--------------------|--------------|
| PSMB7, j2 | -                      | 12                 | -59.5        | 11                 | 182          | 10                 | -52.6        | 9                  | 140          |
| PSMB8    | j3i                    | 13                 | -97.1        | 14                 | 253          | 8                  | -63.4        | 4                  | 157          |
| PSMB9    | j1i                    | 12                 | -74.2        | 11                 | 227          | 6                  | -33.1        | 5                  | 143          |
| PSMB6    | j1                    | 9                  | -26.3        | 4                  | 195          | 5                  | -30.7        | 4                  | 123          |
| PSMB5    | j5                    | 3                  | -16.6        | 6                  | 84           | 3                  | -13          | 6                  | 68           |

### Table IV

| Gene name | Protein Ensemble number | Peptides identified | Protein score | Peptides identified | Protein score | Peptides identified | Protein score | Peptides identified | Protein score |
|-----------|------------------------|--------------------|--------------|--------------------|--------------|--------------------|--------------|--------------------|--------------|
| TAP1      | -                      | ENSP00000402316    | -39.3        | -1.7              | -2.2         | n.d.               |              |                   |              |
| TAP2      | -                      | ENSP00000401971    | -18.8        | -10.9             | -2.7         | n.d.               |              |                   |              |
| TAPBP     | Tapasin               | ENSP00000363700    | -13.1        | n.d.              | -3.1         | n.d.               |              |                   |              |
| B2M       | j2 microglobulin       | ENSP00000389906    | 92.6         | -22.6             | -85.8        | n.d.               |              |                   | -64          |
| BiP       | -                      | n.d.               |              |                   |              | n.d.               |              |                   |              |
| HLA-A     | -                      | ENSP00000403575    | -158.6       | n.d.              | -93.8        | n.d.               |              |                   |              |
| HLA-B     | -                      | ENSP00000352656    | n.d.         | -20.6             | n.d.         | n.d.               |              |                   |              |
| HLA-C     | -                      | ENSP00000400410    | n.d.         | -112.4            | n.d.         | n.d.               |              |                   |              |
| HLA-G     | -                      | ENSP00000416649    | n.d.         | n.d.              | -2.3         | n.d.               |              |                   |              |
proteome studies have the potential to not only validate existing data sets, but to also provide novel functional insights. Improving the confidence in observed features by increasing the number of analyses per sample type is a widely accepted strategy in genomics; our data suggest that the same applies to proteomic studies of difficult-to-characterize biological systems such as platelets.

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