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Granzyme A in Human Platelets Regulates the Synthesis of Proinflammatory Cytokines by Monocytes in Aging


Dysregulated inflammation is implicated in the pathobiology of aging, yet platelet–leukocyte interactions and downstream cytokine synthesis in aging remains poorly understood. Platelets and monocytes were isolated from healthy younger (age <45, n = 37) and older (age 65–85, n = 30) adults and incubated together under autologous and nonautologous conditions. Synthesis of inflammatory cytokines by monocytes, alone or in the presence of platelets, was examined. Next-generation RNA-sequencing allowed for unbiased profiling of the platelet transcriptome in aging. Basal IL-8 and MCP-1 synthesis by monocytes alone did not differ between older and younger adults. However, in the presence of autologous platelets, monocytes from older adults synthesized greater IL-8 (41 ± 5 versus 9 ± 2 ng/ml, p < 0.0001) and MCP-1 (867 ± 150 versus 216 ± 36 ng/ml, p < 0.0001) than younger adults. Platelets from older adults were sufficient for upregulating the synthesis of inflammatory cytokines by monocytes. Using RNA-sequencing of platelets followed by validation via RT-PCR and immunoblot, we discovered that granzyme A (GrmA), a serine protease not previously identified in human platelets, increases with aging (~9-fold versus younger adults, p < 0.05) and governs increased IL-8 and MCP-1 synthesis through TLR4 and caspase-1. Inhibiting GrmA reduced excessive IL-8 and MCP-1 synthesis in aging to levels similar to younger adults. In summary, human aging is associated with changes in the platelet transcriptome and proteome. GrmA is present and bioactive in human platelets, is higher in older adults, and controls the synthesis of inflammatory cytokines by monocytes. Alterations in the platelet molecular signature and signaling to monocytes may contribute to dysregulated inflammatory syndromes in older adults. The Journal of Immunology, 2018, 200: 000–000.

Platelets are anucleate cells with long-established roles central to hemostasis initiation and vascular wall repair. Initially thought to be merely circulating cell fragments with a relatively fixed repertoire of functional responses, platelets are increasingly recognized to be versatile effector cells that bridge thrombotic, inflammatory, and immune continuums (1, 2). Activated platelets stably adhere to and tether monocytes via P-selectin/P-selectin glycoprotein ligand 1 (PSGL-1) and, in parallel, secrete RANTES from platelet α granules. RANTES then binds to CCL5 on monocytes, driving downstream synthesis of proinflammatory gene products by monocytes (3, 4).

Thromboembolic events remain the most common cause of morbidity and mortality in older adults (5) and dysregulated platelet functions in aging are thought to contribute to this heightened thrombosis risk (6), but remain under studied. Thrombosis and inflammation are centrally linked and injurious inflammation is central to the pathobiology of aging. For example, aging is associated with elevated levels of IL-6, IL-8, and C-reactive protein (7, 8). IL-6 has been implicated in mediating thrombosis during systemic inflammatory insults (9, 10). Increased levels of IL-6, IL-8, and MCP-1 during aging may contribute to adverse outcomes in older adults (11–13).

Whereas classic platelet hemostatic functions have been examined in aging, age-associated alterations in the platelet transcriptome and proteome and their effects on platelet–monocyte signaling events have not previously been examined. In this study, we examined

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whether the platelet molecular signature was altered in older adults and dissected a previously unrecognized mechanism whereby platelet–monocyte interactions drive excessive inflammation in aging.

Materials and Methods

Human subjects

The University of Utah Institutional Review Board approved this study (#00051506) and all subjects provided informed consent. Healthy younger (aged <45 y) and older (≥65 y) adults were eligible for participation and recruited through advertising fliers approved by the Institutional Review Board. All study procedures and data capture were done in accordance with ethical regulations and patient data were anonymized. The age cut-off for older adults was based on commonly accepted definitions of aging, and the age cut-off for younger adults was chosen to give sufficient age separation between groups. Age cut-offs were all chosen a priori. Subjects were excluded from the study if they were pregnant (self-reported), had received a blood transfusion within the last 30 d, or had a history of cardiopulmonary disease (including myocardial infarction, arrhythmia, chronic obstructive pulmonary disease, or asthma), infection within the past 30 d, inherited platelet disorder, cancer (whether active or in remission), venous or arterial thromboembolic disease, liver or renal disease, or diabetes. Subjects taking clonidine, dipyridamole, selective serotonin reuptake inhibitors, and phosphodiesterase inhibitors at any dose or frequency were excluded. Subjects refrained from taking nonsteroidal anti-inflammatory drugs for 4 wk prior to study participation. Aspirin was not an exclusionary criterion as many older adults take aspirin for the prevention of cardiovascular disease and stroke. When approved by subjects’ medical providers, aspirin was temporarily discontinued for 4 wk prior to study participation.

Platelet and monocyte isolation

Human peripheral venous blood was drawn into acid citrate dextrose (1.4 ml per 8.6 ml blood) through standard venipuncture technique and used immediately upon collection. The whole blood was first centrifuged at 150 × g for 20 min at 20˚C to separate platelet-rich plasma from RBCs and WBCs. From the platelet-rich plasma, platelets were leukocyte reduced and isolated as previously described (14–17) to yield a highly purified population of cells. Isolated platelets were resuspended in serum-free M199 (Lonza, Walkersville, MD) medium in round-bottom polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ). The purity of isolated platelets was confirmed by flow cytometry and direct counting via a hemocytometer, when <3 leucocytes were observed in each preparation containing 1 × 107 platelets (>99.9% purity, Supplemental Fig. 1A). The number of leucocytes in each preparation did not vary by age (data not shown).

For the isolation of monocytes, the RBC/WBC mixture was resuspended with 0.9% sterile saline back to the original volume and layered over an equal volume of Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway, NJ). The layered cells were then centrifuged for 30 min at 400 g at 20˚C. The cell pellet was then resuspended in MEM (Sigma-Aldrich, St. Louis, MO) with 1% human serum albumin (HSA, BioWhittaker, Walkersville, MD) and counted. The purity of monocytes was confirmed by flow cytometry and direct counting via a hemocytometer, when <3 leucocytes were observed in each preparation containing 1 × 107 platelets (>99.9% purity, Supplemental Fig. 1B).

Flow cytometry

The expression of monocyte surface markers, platelet surface adhesion molecules (e.g., P-selectin), and platelet–monocyte aggregation (PMA) was evaluated by flow cytometry (13, 18–20). All Abs were from BD Biosciences. Isolated, unstimulated monocytes were costained for CD14 FITC and CD16 PE with appropriate isotype controls. Platelet surface P-selectin expression and the formation of PMAs were evaluated as before (13, 18–20). Briefly, whole blood was left alone (baseline) or stimulated with the PAR1 agonist SFLLRN for 10 min at 37˚C (5 μM for P-selectin and 15 μM for PMAs). Whole blood was then incubated with Abs against P-selectin (CD62p), CD14 FITC, or CD41 PE for 20 min at room temperature. Samples were then immediately fixed and run using a FACSscan (BD Biosciences) with appropriate isotype controls. Samples were analyzed using FlowJo v9 (TreeStar, OR). For whole blood platelet activation studies, platelets were gated by forward and side scatter using a logarithmic scale followed by specific monocyte Ag staining compared with isotype control. For monocyte studies, cells were gated by forward and side scatter using a linear scale followed by specific monocyte Ag staining compared with isotype controls. A gating strategy for measuring the percentage of platelets expressing surface P-selectin (CD62p) in whole blood is shown in Supplemental Fig. 1C–E.

Platelet and monocyte incubations

Monocytes (2 × 106 cells per ml, final concentration) and platelets (2 × 108 cells per ml, final concentration) were incubated either separately or together, depending on experimental conditions, at 37˚C for 18 h, as before (3). Cell-free supernatants were then harvested by centrifugation. In select experiments, human recombinant granzyme A (GrmA) (R&D Systems, Minneapolis, MN) was added (100 nM, final concentration) to incubating cells. Platelets were preincubated with an anti-GrmA Ab (R&D Systems) or control IgG (10 μg/ml, final concentration) that specifically blocks GrmA for 1 h, before the addition of monocytes. To identify the cognate receptors regulating cytokine synthesis, monocytes were preincubated with the specific TLR4 inhibitor, CLI-095 (1 μM, final concentration; InvivoGen, San Diego, CA) or a specific WEHD-FMK caspase-1 inhibitor (1 μM, final concentration; R&D Systems) for 1 h.

Chemokine and cytokine protein expression

Platelet chemokines (e.g., P-selectin, RANTES, platelet factor 4 (PF4)) and monocyte chemokines (IL-6, IL-8, and MCP-1) were measured by ELISA (R&D Systems) per the manufacturer’s instructions. For determination of chemokines in cell lysates, platelets (2 × 106 cells per ml, final concentration) were lysed in radioimmunoprecipitation assay buffer. P-selectin, RANTES, and PF4 were measured by ELISA (R&D Systems) per the manufacturer’s instructions.

Next-generation RNA-sequencing and RNA expression

Highly purified platelets were isolated as described above. For next-generation RNA-sequencing (RNA-seq), isolated platelets were carefully lysed in Trizol, and DNase-treated total RNA was isolated, as previously described (14, 16, 21–23). An Agilent bioanalyzer was used for quality control and to quantitate RNA. RNA Integrity Number scores were similar between all samples (data not shown). RNA-seq libraries were prepared with TruSeq V2 with oligo-dT selection (Illumina, San Diego, CA). Reads were aligned (Novoalign) to the reference genome GRC37/hg19 and a pseudo-transcriptome containing splice junctions. The Deseq2 analysis package was used to assign reads to composite transcripts (one per gene) and quantitate fragments per kilobase of transcript per million mapped reads (FPKM) as previously described (24). The expression of candidate transcripts identified by RNA-seq was further examined using quantitative RT-PCR (qRT-PCR). Forward and reverse primers were as follows, respectively: 1) GrmA: 5’-CATTTGATGTGGTGGGACA-3’, 5’-TCTGGATTCTTGTTGCCAG-3’, 2) GrmH: 5’-GCTTCTCTGA-GAAAATGCAG-3’, 5’-GAGCAAGTCTGACAAAGAAG-3’, 3) GrmM: 5’-AGCTGAGCGGGGAAGTGGA-3’, 5’-CCAGAGGCCTGTGGT-TAC-3’, 4) Granulysin: 5’-GATGAGGCTGCTGAAGAGTC-3’, 5’-GTGAGGGAGGTTTGGTAGA-3’.
cytometry. m with thrombin-receptor activating peptide (TRAP: 5 μM for CD41/CD14, 15 μM for P-selectin) prior to Ab staining and assessment by flow cytometry. *p < 0.05.

Immunocytochemistry

Freshly isolated platelets were fixed in suspension with paraformaldehyde (2% final concentration), placed in chamber slides, and subsequently incubated with IgG or a specific Ab against GrmA (1:100 dilution, 60 min; (2% final concentration), placed in chamber slides, and subsequently in

Freshly isolated platelets were fixed in suspension with paraformaldehyde (Shandon Cytospin; Thermo Fisher Scientific, Waltham, MA). Fluorescence microscopy was performed using an Olympus IX81, FV300 (Olympus, Melville, NY) confocal-scanning microscope equipped with a 60×/1.42 NA oil objective for viewing platelets. An Olympus FVS-PSU/IX2-UCB camera and scanning unit and Olympus Fluoview FV 300 image acquisition software version 5.0 were used for recording.

Protein expression studies

All samples were normalized for starting cell concentrations. The samples were centrifuged at 13,000 × g for 4 min and then resuspended in 0.5 ml of deionized water. The protein was pelleted by centrifugation at 13,000 × g for 4 min. The protein pellets were solubilized in sample buffer containing 0.1 M Tris, 2% SDS, 1% (v/v) glycerol, 0.1% bromophenol blue, and 100 mM DTT then boiled for 10 min. Proteins were resolved by SDS polyacrylamide gels and transferred to nitrocellulose membrane (Whatman Portran). Membranes were blocked with Odyssey blocking buffer for 1 h at ambient temperature, incubated overnight at 4˚C with the desired primary Ab for GrmA (Santa Cruz Biotechnology), and then washed four times with TBST. Membranes were then incubated with appropriate secondary infrared dye-labeled Ab for 60 min at room temperature and washed four times with TBST. Membranes were examined and quantified with a Li-Cor Odyssey infrared imaging system. In select experiments, platelets were stimulated with thrombin for the appropriate time under stirring conditions at 37˚C, and the reaction was stopped by the addition of 0.6 NHClO4.

Statistical methods

For RNA-seq analysis, Deseq2 was used to identify differentially expressed transcripts, as we have previously described (24). To focus on robustly expressed transcripts, only transcripts with per-group average ≥3 FPKM were included in the analysis. RNAs ≥2-fold differentially expressed between older and younger adults and with a nominal p value <0.05 (for discovery) were included in the analysis. To make these data publicly available, a complete dataset of the RNA-seq studies was uploaded to Gene Expression Omnibus (SRA2797948). For relevant studies, we calculated the mean ± SEM and performed ANOVA to identify differences among multiple experimental groups. If significant differences existed, a Student Newman–Keuls post hoc procedure was used to determine the location of the difference between groups. When single comparisons were performed, data were tested for normality and skewness and a Student t test or Wilcoxon rank sum was employed, as appropriate. Statistical significance was set at a two-tailed p value <0.05.

Results

Platelets from older adults enhance IL-8 and MCP-1 synthesis

Table I shows the clinical characteristics of the study cohort. The two groups [younger (age <45 y) and older (age ≥65 y)] were well balanced with regards to gender. Older adults had a higher

Immunochemistry

Aged platelets enhance IL-8 and MCP-1 synthesis. Monocytes and platelets were isolated from younger (age <45 y, n = 27) or older adults (age ≥65 y, n = 27). (A and B) Monocytes alone were incubated in the presence or absence of thrombin (IIa, 0.1 U/ml) for 18 h. IL-8 and MCP-1 synthesis by monocytes alone did not significantly increase with thrombin and was similar between younger and older adults. (C and D) Isolated monocytes were incubated with thrombin activated, autologous platelets, or nonautologous (switch) platelets. IL-8 and MCP-1 synthesis was significantly increased in the presence of platelets from older adults. In comparison, in switch experiments, when monocytes from an older adult were incubated with platelets from a younger adult, IL-8 and MCP-1 synthesis was significantly decreased. *p < 0.05.

FIGURE 1. Aged platelets enhance IL-8 and MCP-1 synthesis. Monocytes and platelets were isolated from younger (age <45 y, n = 27) or older adults (age ≥65 y, n = 27). (A and B) Monocytes alone were incubated in the presence or absence of thrombin (IIa, 0.1 U/ml) for 18 h. IL-8 and MCP-1 synthesis by monocytes alone did not significantly increase with thrombin and was similar between younger and older adults. (C and D) Isolated monocytes were incubated with thrombin activated, autologous platelets, or nonautologous (switch) platelets. IL-8 and MCP-1 synthesis was significantly increased in the presence of platelets from older adults. In comparison, in switch experiments, when monocytes from an older adult were incubated with platelets from a younger adult, IL-8 and MCP-1 synthesis was significantly decreased. *p < 0.05.

FIGURE 2. Aging does not alter monocyte CD14 or CD16 surface expression, platelet surface P-selectin expression, or the formation of platelet-monocyte aggregates. Whole blood was drawn from younger (age <45 y) and older (age ≥65 y) subjects. Blood was stained for surface monocyte markers CD14 or CD16 [(A), n = 6 younger and n = 6 older], surface adhesion platelet P-selectin marker CD62P [(B), n = 23 younger and n = 13 older], or platelet–monocyte aggregates as measured by double positivity for CD41 and CD14 [(C), n = 26 younger and n = 14 older]. Expression was adjusted for nonspecific staining by using appropriate isotype controls. In (B and C), whole blood was fixed immediately (baseline, BL) or activated with thrombin-receptor activating peptide (TRAP: 5 μM for CD41/CD14, 15 μM for P-selectin) prior to Ab staining and assessment by flow cytometry. *p < 0.05.
body mass index and were taking aspirin more commonly (Table I).

When monocytes were allowed to incubate alone (i.e., in the absence of platelets), basal IL-8 and MCP-1 protein levels in the supernatant were negligible and did not differ between younger and older adults (Fig. 1A, 1B). In comparison, when monocytes were coincubated with autologous platelets (i.e., monocytes and platelets from the same subject), IL-8 and MCP-1 synthesis increased robustly in both groups but was significantly higher (∼4-fold) in older adults compared with younger adults (Fig. 1C, 1D). In older subjects, the synthesis of IL-8 and MCP-1 did not significantly differ between aspirin users and nonusers (IL-8: 35.8 ± 14.8 versus 24.9 ± 6.7 ng/ml, MCP-1: 1105 ± 267 versus 710 ± 309 ng/ml). We also observed increased IL-6 synthesis in older adults, when cocultured with autologous platelets, as compared with younger subjects, but the differences did not meet statistical significance (Supplemental Fig. 2A).

To determine whether platelets were the cellular drivers of the increased synthesis of IL-8 and MCP-1 in older adults, we next performed switch experiments. In these switch experiments, monocytes from an older adult were coincubated with platelets isolated from a gender-matched younger adult (e.g., nonautologous conditions). In parallel, monocytes from a younger adult were coincubated with platelets from an older adult. In all these experiments, monocytes and platelets from younger or older adults were isolated simultaneously and experiments performed on the same day in parallel. As shown in Fig. 1C and 1D, IL-8 and MCP-1 synthesis by monocytes from older adults was rescued when coincubated with platelets from younger adults. Conversely, IL-8 and MCP-1 synthesis by monocytes from younger adults was significantly enhanced when coincubated with platelets from older adults. Synthesis of IL-8 by young monocytes was similar to that seen in older monocytes, when coincubated with aged platelets.

FIGURE 3. Aging does not alter platelet expression or secretion of the adhesion molecule P-selectin, the signaling molecule RANTES, or the monocyte chemotactic molecule PF4. (A) Platelets were freshly isolated from younger (age <45 y, n = 3) and older subjects (age ≥65 y, n = 3). Total RNA was isolated from platelets and the platelet transcriptome was assessed by next-generation RNA-seq. The total RNA expression of P-selectin, RANTES, and PF4 was examined in RNA-sequenced, highly purified platelet lysates. Shown are representative Integrative Genomics Viewer browser images of each transcript in platelets from a younger (top panels, blue) and older (bottom panels, red) adult with quantified transcript FPKM levels on the right (FPKM: n = 3 per group). The y-axis represents the relative expression of each mRNA, with higher peaks indicating increased expression. (B–D) To determine the protein expression, platelets were isolated from younger (age <45 y, n = 11) and older subjects (age ≥65 y, n = 11). Platelets were either immediately lysed (baseline, BL) or activated with thrombin (IIa, 0.1 U/ml, t = 30 min). Platelet supernatants were harvested by centrifugation. Total P-selectin (B), RANTES (C), and PF4 (D) protein levels were measured in platelets lysates (for total protein) and in supernatants (Sups) by ELISA.
These results indicate that aged platelets are responsible for triggering increased IL-8 and MCP-1 produced by monocytes in older adults. The expression of cell-surface adhesion molecules and the secretion of platelet chemokines that regulate IL-8 and MCP-1 synthesis are not altered in aging.

Induction of IL-8 and MCP-1 synthesis by monocytes requires stable adhesion of platelets to monocytes, primarily through engagement of P-selectin on the platelet surface to PSGL-1 on monocytes (3). Thus, we sought to determine whether the expression of monocyte and platelet surface adhesion or signaling molecules was increased in our cohort of older adults. We did not identify any differences in basal or activation-dependent expression of platelet surface P-selectin, platelet–monocyte aggregate formation, or the number of monocytes positive for CD14+ or CD16+ between younger and older adults (Fig. 2).

We next examined whether aging was associated with changes in the mRNA levels, intracellular expression, or secretion of proteins required for platelet–monocyte interactions and downstream signal-dependent cytokine synthesis. In unstimulated platelets, there was no difference in the basal mRNA or intracellular protein expression of P-selectin and RANTES (required for platelet signaling to monocytes and cytokine synthesis), or PF4 [a chemotactic factor for monocytes (25)] between younger and older adults (Fig. 3). Similarly, the secretion of P-selectin, RANTES, or PF4 by activated platelets was similar between younger and older adults (Fig. 3). Together, these findings suggest that the increased IL-8 and MCP-1 synthesis by monocytes from older adults was not due to enhanced expression of these platelet surface adhesion and signaling molecules. Accordingly, we turned our attention to heretofore unidentified proteins in human platelets with the capacity for regulating proinflammatory gene synthesis by monocytes.

**FIGURE 4.** GrmA is present in human platelets and its expression increases with aging. (A) Platelets were isolated from younger (n = 3) and older (n = 3) adults. Total RNA was isolated from platelets and the platelet transcriptome was interrogated using next-generation RNA-seq. All transcripts identified by RNA-seq were then filtered to identify only those that were differentially expressed (log2 fold-change ≥2). The heat map illustrates only those differentially expressed (log2 fold-change ≥2), upregulated (red), and downregulated (blue) transcripts that were identified in platelets from older adults as compared with younger adults. (B) Representative Integrative Genomics Viewer browser images of GrmA transcript expression in platelets isolated from a younger (top, blue) and older (bottom, red) adult. The y-axis represents the relative expression of GrmA, with higher peaks indicating increased total mRNA expression. On the bottom, the thick bars on the x-axis illustrate the exons and the 5′ and 3′ ends of the transcript are annotated (left and right, respectively). (C) GrmA expression was quantified by qRT-PCR in platelets isolated from older and younger adults. GrmA expression, but not GrmH, GrmM, or granulysin expression, was significantly increased in older adults. (D) Protein expression of GrmA was measured in isolated platelets (5 × 10^7 platelets equally loaded for each subject) by immunoblot and quantified by densitometry in older (n = 7) and younger (n = 7) adults. The left panel illustrates a representative immunoblot from n = 4 younger subjects and n = 3 older subjects with actin as a loading control in the bottom. The right panel shows normalized quantification of GrmA protein expression. *p < 0.05 versus younger adults.
GrmA is expressed in human platelets and increases with aging

To globally interrogate the platelet transcriptome and identify other molecules governing enhanced cytokine synthesis during aging, we next performed RNA-seq on isolated platelets from a subset of younger (n = 3) and older (n = 3) adults. We identified numerous (n = 514) transcripts significantly differentially expressed with aging, with most being increased in older adults (455 mRNAs increased and 59 mRNAs decreased; Fig. 4A, Supplemental Tables I, II). Among the differentially expressed candidates, we focused on GrmA, a serine protease that governs proinflammatory responses and protein synthesis by monocytes that, in some settings, may be perforin independent (26, 27). By RNA-seq, GrmA expression was robustly increased in platelets from older adults as compared with younger adults (Fig. 4B). Subsequent interrogation of GrmA expression by qRT-PCR on a larger cohort confirmed that platelet GrmA expression was significantly increased in older adults (Fig. 4C). The expression of GrmH, GrmM, and granulysin did not differ between younger and older adults (Fig. 4C), GrmA protein expression was also significantly increased in platelets from older adults (Fig. 4D). Immunofluorescence studies confirmed the presence of GrmA protein within human platelets in submembranous areas and in a granular pattern that did not appreciably colocalize with α granules (Fig. 5A). When platelets from younger or older adults were activated with thrombin, GrmA was almost entirely secreted into the extracellular milieu (Fig. 5B). Thus, GrmA in platelets is under signal-dependent secretion and released extracellularly (where it may signal to monocytes). GrmA secretion was higher in platelets from older adults as compared with platelets from younger adults (Fig. 5B).

GrmA increases IL-8 and MCP-1 synthesis

To examine the role of GrmA in potentiating IL-8 and MCP-1 synthesis by monocytes, we next incubated monocytes and platelets with recombinant human GrmA (rhGrmA), at a concentration (100 nM) similar to that used by other investigators in prior publications (28, 29). When either monocytes or platelets were incubated alone in the presence or absence of rhGrmA, IL-8, and MCP-1 synthesis did not significantly increase (Fig. 5C, 5D). These data indicate that under these experimental conditions and in the absence of platelets, GrmA is insufficient to trigger appreciable IL-8 and MCP-1 synthesis. However, when monocytes were coincubated with platelets in the presence of rhGrmA, IL-8, and MCP-1 synthesis was significantly upregulated (Fig. 5C, 5D). We did not identify GrmA in resting human monocytes (Fig. 6A). As a positive control, we confirmed that the TLR4 inhibitor blocked LPS-induced synthesis of IL-8 by resting monocytes (Fig. 6B). The addition of polymyxin B, which blocks LPS-induced activation of TLR4, did not prevent GrmA-induced cytokine synthesis, indicating that LPS contamination was not causing the observed increase in IL-8 and MCP-1 synthesis (Supplemental Fig. 2B, 2C). Inhibition of GrmA, using a specific anti-GrmA Ab, reduced cytokine production to levels similar to conditions where GrmA was absent (Fig. 6C). Control IgG had no effect on IL-8 or MCP-1 synthesis, indicating that this reduction of GrmA-induced synthesis of IL-8 and MCP-1 is independent of FcγR Ab binding (Fig. 6C). Taken together, these findings identify that platelet GrmA controls the synthesis of IL-8 and MCP-1 protein by monocytes.
Inhibiting GrmA in older adults rescues IL-8 and MCP-1 synthesis through TLR4 and caspase-1–dependent mechanisms

We next sought to determine if inhibiting GrmA in platelets from older adults would normalize (to levels seen in younger adults) IL-8 and MCP-1 synthesis by monocytes. To establish this, platelets and monocytes from older adults were coincubated in the presence or absence of an anti-GrmA Ab. When GrmA was blocked, the synthesis of IL-8 and MCP-1 was reduced to levels similar to those seen in younger adults (Fig. 7A).

To dissect the mechanisms of action whereby GrmA was inducing cytokine synthesis, we next measured GrmA-induced IL-8 and MCP-1 cytokine synthesis in the presence or absence of specific inhibitors to TLR4 or caspase-1. In younger adults, GrmA-induced production of IL-8 and MCP-1 was blocked completely when TLR4 was inhibited (Fig. 7B). In contrast, when caspase-1 was inhibited, we observed partial rescue of IL-8 whereas MCP-1 synthesis was completely blocked (Fig. 7B). Similar findings were observed in older adults (Fig. 7C). TL4 mRNA and protein expression in either isolated platelets or monocytes did not differ between younger and older subjects (Fig. 7D–G). Consistent with this, gene ontology analyses also did not identify any differences in the TLR pathway in platelets based on RNA-seq data (data not shown).

Discussion

Platelets are circulating anucleate blood cells traditionally thought to have a relatively fixed transcriptome and proteome, and have rapid, short-lived functions primarily for hemostasis and wound repair (2). Emerging evidence indicates, however, that platelets possess a broad and dynamic repertoire of functions (2, 30–32). Platelets process precursor mRNAs in response to activating signals, synthesize new proteins, and have activities that span inflammatory and immune continuums (16, 32).

Aging is associated with injurious thromboinflammation and alterations in platelet functions (33–35). In older adults with acute, systemic inflammatory syndromes, increased platelet activation correlates with increased circulating, proinflammatory cytokine levels, and adverse clinical outcomes (13). Circulating plasma levels of IL-6 increase with age (11, 12) and monocytes from older adults express greater intracellular IL-6 and IL-8 (8). MCP-1 is chemotactic for mononuclear leukocytes into inflamed vascular tissues and IL-8 orchestrates firm adhesion of monocytes to vascular endothelium (36). As we and others have shown, the formation of circulating platelet–monocyte aggregates is increased in older adults with systemic inflammatory syndromes (13) and PMA results in the synthesis of IL-8 and MCP-1 (3). Stable adhesion of platelets to monocytes requires the expression of P-selectin on the platelet surface and subsequent engagement of PSGL-1 on the monocyte. The release of the platelet α granule protein RANTES (through binding to CCR5 on the monocyte surface) causes translocation of NF-κB into the nucleus of the monocyte and triggers the synthesis of IL-8 and MCP-1 (3).

In this study, using both autologous and nonautologous monocytes (switch co-culture conditions), we demonstrate that aged platelets drive excessive production of IL-6, IL-8, and MCP-1 by...
monocytes. We identify that, molecularly, platelet GrmA mediates the heightened synthesis of these proinflammatory cytokines and that platelet GrmA is significantly enriched (at both the mRNA and protein level) and bioactive in older adults. Inhibiting GrmA in vitro in older adults reduced IL-8 and MCP-1 synthesis to levels comparable to those in younger adults. Importantly, we confirmed that our findings were not due to any inadvertent contamination by LPS. We could not find any prior studies that identified GrmA in human platelets. This is also the first evidence, to our knowledge, that shows where aging-associated increases in GrmA are sufficient to drive proinflammatory gene synthesis by monocytes.

These findings build upon and extend our understanding of the cellular expression and function of GrmA. In humans, granzymes are a family of five structurally related serine proteases found ubiquitously in cytotoxic lymphocytes that differ in their substrate specificity (27, 37). GrmA was initially identified within cytoplasmic granules within cytotoxic T cells and NK cells (38). More recently, GrmA has been found within other nucleated cells and in the extracellular space (39), been shown to activate macrophages, monocytes, and mast cells, and induces inflammatory responses independent of perforins (26, 27).

Our studies also provide new evidence that GrmA functionally regulates the production of cytokines by monocytes. Although we focused on elucidating the mechanism of excessive cytokine production in aging, our data demonstrate that even in younger adults, modulating GrmA (either by addition of exogenous GrmA or by inhibiting endogenous GrmA) serves to control IL-8 and MCP-1 synthesis. Moreover, inhibition of TLR4 or caspase-1 reduced cytokine synthesis in response to GrmA. These findings are consistent with prior reports showing that GrmA enhances activation of the inflammasome (a caspase-1–dependent event) and LPS-mediated signaling, which acts via TLR4 (29). In addition, mice globally deficient in GrmA exhibit better survival in response to a lethal LPS challenge, as compared with wild-type mice where GrmA is endogenously present (29, 40).

The strengths of our study include the use of freshly isolated, primary human cells (e.g., platelets and monocytes), our approximation of physiological conditions when incubating platelets and monocytes together, and our rigorous validation of the expression and activity of GrmA in younger and older human participants. We have also made all the platelet RNA-seq data publicly available. Fastq files have been submitted to the Sequence Read Archive so that readers and investigators may query the data directly (National Center for Biotechnology Information BioProject PRJNA397446, accession numbers SRR5907423–SRR5907428). The BioProject can be accessed at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA397446.

Nonetheless, whether GrmA is acting specifically and selectively on monocytes, platelets, or both remains to be determined. Although human platelets are not known to express or synthesize IL-8 and MCP-1 protein, they do express TLR4 on their surface and have a functional inflammasome (22, 41–43). Thus, altered signaling through these pathways may influence how platelets interact with monocytes, leading to increased monocyte-driven cytokine synthesis in older adults. Platelets are increasingly recognized as effector cells during systemic, injurious inflammatory responses. Our data support established and emerging investigations examining
whether antiplatelet therapies modulate cytokine synthesis (and thus inflammation). For example, dipryidamole, but not aspirin, attenuates nuclear translocation of NF-kB and MCP-1 synthesis (44). This may explain in part why the combination of aspirin plus extended-release dipryidamole offered better secondary stroke risk reduction than aspirin alone in clinical trials (45, 46). Whether targeting platelets in other inflammatory diseases offers clinical benefits remains an active area of study.

Although not a central focus of our study, our findings also demonstrate that the platelet transcriptome is altered in aging. We identified numerous differentially expressed transcripts in platelets isolated from older adults with enrichment of pathways implicated in cell-cell signaling and inflammatory pathways (data not shown). Our age-related changes in the platelet transcriptome were similarly noted in a younger cohort of healthy patients aged 18–46 y, where more than 120 mRNAs and 15 microRNAs demonstrated age-dependent expression levels (47). We extend this published work by offering, to our knowledge, the first human platelet RNA-seq dataset comparing younger (age <45 y) and older (age ≥65 y) individuals. We have made this dataset available and hope it will serve as a discovery tool for investigators in the field. In conclusion, human aging is associated with changes in the platelet transcriptome and proteome. GrmA is present and bioactive hope it will serve as a discovery tool for investigators in the field. (age

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Aging Cell


Aging is associated with greater nuclear NF kappa B, reduced I kappa B alpha, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. Aging Cell 7: 805–812.


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