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Granzyme A in Human Platelets Regulates the Synthesis of Pro-Inflammatory Cytokines by Monocytes in Aging

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Abstract

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, n=30) adults and incubated together under autologous and non-autologous 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 vs. 9±2 ng/mL, p<0.0001) and MCP-1 (867±150 vs. 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-seq 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, is increased in aging (~9-fold vs. 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

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Disclosures

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and signaling to monocytes may contribute to dysregulated inflammatory syndromes in older adults.

MESH Keywords

Granzymes; Aging; Blood Platelets; Monocytes; Inflammation; Genes

Introduction

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/PSGL-1 (P-selectin glycoprotein ligand 1) and in parallel, secrete regulated on activation, normal T cell expressed and secreted (RANTES) from platelet alpha granules. RANTES then binds to CCL5 on monocytes, driving downstream synthesis of pro-inflammatory 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 understudied. 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 interleukin(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 monocyte chemotactic protein 1 (MCP-1) during aging may contribute to adverse outcomes in older adults[11–13].

While 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. Here, we examined 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 (IRB # 00051506) and all subjects provided informed consent. Healthy younger (defined as age<45 years) and older (defined as age≥65 years) were eligible for participation and were recruited through advertising flyers approved by the IRB. All study procedures and data capture was done in accordance with ethical regulations and patient data was 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 any blood transfusion within the last 30 days or had a history of

cardiopulmonary disease (including myocardial infarction, arrhythmia, chronic obstructive pulmonary disease, or asthma), infection within the past 30 days, inherited platelet disorder, cancer (whether active or in remission), venous or arterial thromboembolic disease, liver or renal disease, or diabetes. Subjects taking clopidogrel, dipyridamole, selective serotonin reuptake inhibitors (SSRIs), and phosphodiesterase inhibitors at any dose or frequency were excluded. Subjects refrained from taking non-steroidal anti-inflammatory drugs (NSAIDs) for 4 weeks 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 subject' medical provider, aspirin was temporarily discontinued for 4 weeks prior to study participation.

Platelet and monocyte isolation

Human peripheral venous blood was drawn into acid-citrate-dextrose (1.4 ml ACD/8.6 ml blood) through standard venipuncture technique and used immediately upon collection. The whole blood was first centrifuged at 150 x g for 20 min at 20°C to separate platelet-rich plasma (PRP) from red and white blood cells (RBC/WBC). From the PRP, platelets were leukocyte-reduced and isolated as previously described[14–17] to yield a highly-purified population of cells. Isolated platelets were resuspended at in serum-free M199 (Lonza, Walkersville, USA) medium in round-bottom polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ). The purity of isolated platelets was confirmed by flow cytometry and also direct counting via a hemocytometer, where less than 3 leukocytes were observed in each preparation containing 1x10^7 platelets (>99.9% purity, Supplemental Figure 1A). The number of leukocytes in each preparation did not vary by age (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, USA). The layered cells were then centrifuged for 30 min at 400 x g at 20°C. After 30 minutes, the mononuclear leukocyte layer was removed and washed with Hank's Balance Salt Solution (HBSS: Sigma-Aldrich, St. Louis, MO, USA) with 1% human serum albumin (HBSS/A; University of Utah Hospital, Salt Lake City, UT, USA) and centrifuged for 10 min at 400 x g at 20°C. The cell pellet was then resuspended in HBSS/A and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 15 min at 4°C. The cells were then washed with HBSS/A to remove any free CD14 microbeads and then resuspended in HBSS/A. The monocytes were then isolated by running the suspended cells through an autoMACs cell separator (Miltenyi Biotec) using the PosselD2 program. The monocytes were washed with HBSS/A and resuspended in M199 (BioWhitaker, Walkersville, MD, USA) and counted. The purity of monocytes was confirmed to be > 99.4% by flow cytometry (Supplemental Figure 1B).

Flow cytometry

The expression of monocyte surface markers, platelet surface adhesion molecules (e.g. P-selectin), and platelet-monocyte aggregation (PMAs) was evaluated by flow cytometry[13, 18–20]. All antibodies were from BD Biosciences. Isolated, unstimulated monocytes were co-stained for CD14 fluorescein isothiocyanate (FITC) and CD16 phycoerythrin (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 minutes at 37°C (5μM for P-selectin and 15μM for PMAs). Whole blood was then incubated with antibodies against P-selectin (CD62p), CD14 FITC, or CD41 PE for 20 minutes at room temperature. Samples were then immediately fixed and run using a FACScan (BD Biosciences, USA) with appropriate isotype controls. Samples were analyzed using FlowJo v9 (Oregon, USA). For whole blood platelet activation studies, platelets were gated by forward and side scatter using a logarithmic scale followed by specific platelet antigen staining compared to isotype control. For monocytes studies, cells were gated by forward and side scatter using a linear scale followed by specific monocyte antigen staining compared to isotype controls. A gating strategy for measuring the percentage of platelets expressing surface P-selectin (CD62p) in whole blood is shown in Supplemental Figures 1C–E.

Platelet and Monocyte Incubations

Monocytes ($2x10^6$ cells/mL, final concentration) and platelets ($2x10^8$ cells/mL, final concentration) were incubated either separately or together, depending on experimental conditions, at 37°C for 18 hours, as before[3]. Cell free supernatants were then harvested by centrifugation. In select experiments, human recombinant GrmA (R&D systems, Minneapolis, Minnesota) was added (100nM, final concentration) to incubating cells. Platelets were pre-incubated with an anti-GrmA antibody (R&D systems, Minneapolis, Minnesota) or control IgG (10μg/mL, final concentration) that specifically blocks GrmA for one hour, before the addition of monocytes. To identify the cognate receptors regulating cytokine synthesis, monocytes were pre-incubated with the specific toll-like receptor 4 (TLR4) inhibitor, CLI-095 (1μM, final concentration; Invivogen, San Diego, California) or a specific WEHD-FMK Caspase-1 inhibitor (1μM, final concentration; R&D systems, Minneapolis, Minnesota) for one hour.

Chemokine and Cytokine Protein Expression

Platelet chemokines (e.g. P-selectin, RANTES, PF4) and monocyte cytokines (IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1) were measured in harvested supernatants by commercially available ELISA (R&D Systems, Minneapolis, Minnesota) per the manufacturer's instructions. For determination of chemokines in cell lysates, platelets ($2x10^8$ cells/mL, final concentration) were lysed in RIPA buffer. P-selectin, RANTES, and platelet factor 4 (PF4) were measured by ELISA (R&D Systems, Minneapolis, Minnesota) 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 bio-analyzer was used to Quality Control (QC) and quantitate RNA. RNA Integrity Number (RIN) scores were similar between all samples (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 GRCh37/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 FPKMs 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′-CATTGATTGATGTGGGGACA-3′, 5′-TCTGGGATTTCTGGTTCAGG-3′; (2) *GrmH*: 5′-GCCTTCCTGAGAAAATGCAG-3′, 5′-GAGCAGCTGTCAGCACAAAG-3′; (3) *GrmM*: 5′-AGCTGGACGGGAAAGTGAAG-3′, 5′-CCAGAAGCGGCTGTTGTTAC-3′; (4) *Granulysin*: 5′-GATGAGGCTGCTGAAAGGTC-3′, 5′-GTGGAGGGAGTTTGGTGAGA-3′.

Immunocytochemistry

Freshly-isolated platelets were fixed in suspension with paraformaldehyde (PFA, 2% final concentration), placed in chamber slides, and subsequently incubated with IgG or a specific antibody against GrmA (1:100 dilution, Santa Cruz Biotechnology, Dallas, TX, 60 minutes). Fixed platelets were subsequently layered onto vectabond™ (Vector Laboratories, Burlingame, CA) coated coverslips using a cytospin centrifuge (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 60x/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 x g for 4 minutes and then resuspended in 0.5ml of deionized water. The protein was pelleted by centrifugation at 13,000 x g for 4 minutes. The protein pellets were solubilized in sample buffer containing 0.1 M Tris, 2% SDS, 1% (v/v) glycerol, 0.1% bromophenol blue, and 100mM DTT then boiled for 10 minutes. Proteins were resolved by SDS polyacrylamide gels and transferred to nitrocellulose membrane (Whatman Protran). Membranes were blocked with Odyssey blocking buffer for 1 hour at ambient temperature, incubated overnight at 4°C with the desired primary antibody for GrmA (Santa Cruz Biotechnology, Dallas, TX) and then washed 4 times with TBS-T. Membranes were then incubated with appropriate secondary infrared dye-labeled antibody for 60 minutes at room temperature and washed 4 times with TBS-T. 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 0.6N $HClO_4$.

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 greater than 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 (SRA2779748). 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 was tested for normality and skewness and a Student's 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 1 shows the clinical characteristics of the study cohort. The two groups [younger (age<45 years) and older (age≥65 years)] were well balanced with regards to gender. Older adults had a higher BMI and were taking aspirin more commonly (Table 1). 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 (Figure 1A–B). In comparison, when monocytes were co-incubated 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 to younger adults (Figure 1C–D). In older subjects, the synthesis of IL-8 and MCP-1 did not significantly differ between aspirin users and non-users (IL-8: 35.8±14.8 vs. 24.9±6.7 ng/mL, MCP-1: 1105±267 vs. 710±309 ng/mL). We also observed increased IL-6 synthesis in older adults, when co-cultured with autologous platelets, as compared to younger subjects, but the differences did not meet statistical significance (Supplemental Figure 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 co-incubated with platelets isolated from a gender-matched younger adult (e.g. non-autologous conditions). In parallel, monocytes from a younger adult were co-incubated 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 Figures 1C and 1D, IL-8 and MCP-1 synthesis by monocytes from older adults was rescued when co-incubated with platelets from younger adults. Conversely, IL-8 and MCP-1 synthesis by monocytes from younger adults was significantly enhanced when co-incubated with platelets from older adults. Synthesis of IL-8 by young monocytes was similar to that seen in older monocytes, when co-incubated with aged platelets (Figure 1C). 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 is 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 P-selectin glycoprotein ligand 1 (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 (Figure 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 platelet factor 4 (PF4, a chemotactic factor for monocytes[25]) between younger and older adults (Figure 3). Similarly, the secretion of P-selectin, RANTES, or PF4 by activated platelets was similar between younger and older adults (Figure 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 here-to-fore unidentified proteins in human platelets with the capacity for regulating pro-inflammatory gene synthesis by monocytes.

Granzyme A 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-sequencing 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; Figure 4A and Supplemental Tables 1–2). Among the differentially expressed candidates, we focused on Granzyme A (GrmA), a serine protease that governs pro-inflammatory 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 to younger adults (Figure 4B). Subsequent interrogation of *GrmA* expression by qRT-PCR on a larger cohort confirmed that platelet *GrmA* expression was significantly increased in older adults (Figure 4C). The expression of *Granzyme H* (*GrmH*), *Granzyme M* (*GrmM*) and *granulysin* did not differ between younger and older adults (Figure 4C). GrmA protein expression was also significantly increased in platelets from older adults (Figure 4D). Immunofluorescence studies confirmed the presence of GrmA protein within human platelets in submembraneous areas and in a granular pattern that did not appreciably co-localize with alpha granules (Figure 5A). When platelets from younger or older adults were activated with thrombin, GrmA was almost entirely secreted into the extracellular milieu (Figure 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 to platelets from younger adults (Figure 5B).

Granzyme A 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 (100nM) 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 (Figure 5C–D). 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 co-incubated with platelets in the presence of rhGrmA, IL-8 and MCP-1 synthesis was significantly upregulated (Figure 5C–D). We did not identify *GrmA* in resting human monocytes (Figure 6A). As a positive control, we confirmed that the TLR4 inhibitor blocked LPS-induced synthesis of IL-8 by resting monocytes (Figure 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 Figure 2B–C). Inhibition of GrmA, using a specific anti-GrmA antibody, reduced cytokine production to levels similar to conditions where GrmA was absent (Figure 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 antibody binding (Figure 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 co-incubated in the presence or absence of an anti-GrmA antibody. When GrmA was blocked, the synthesis of IL-8 and MCP-1 was reduced to levels similar to those seen in younger adults (Figure 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 (Figure 7B). In contrast, when caspase-1 was inhibited, we observed partial rescue of IL-8 while MCP-1 synthesis was completed blocked (Figure 7B). Similar findings were observed in older adults (Figure 7C). TL4 mRNA and protein expression in either isolated platelets or monocytes did not differ between younger and older subjects (Figures 7D–G). Consistent with this, gene ontology (GO) analyses also did not identify any differences in the TLR pathway in platelets based on RNA-seq 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 pre-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 thrombo-inflammation and alterations in platelet functions[33–35]. In older adults with acute, systemic inflammatory syndromes, increased platelet activation correlates with increased circulating, pro-inflammatory cytokine levels and adverse clinical outcomes[13]. Circulating plasma levels of IL-6 are increased in aging[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 platelet-monocyte aggregation 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 alpha granule protein RANTES (through binding to CCR5 on the monocyte surface) causes translocation of NF-Kβ into the nucleus of the monocyte and triggers the synthesis of IL-8 and MCP-1[3].

Here, using both autologous and non-autologous 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 pro-inflammatory 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, of where aging-associated increases in GrmA are sufficient to drive pro-inflammatory 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 natural killer (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. While 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 challenged, as compared to wild-type mice where GrmA is endogenously present[29, 40].

The strengths of our study includes 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 sequencing data publicly available. Fastq files have been submitted to the Sequence Read Archive (SRA) so that readers and investigators may query the data directly themselves (NCBI BioProject ID 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. While 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 anti-platelet therapies modulate cytokine synthesis (and thus inflammation). For example, dipyridamole, 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 dipyridamole 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.

While 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 (not shown). Our age-related changes in the platelet transcriptome were similarly noted in a younger cohort of healthy patients aged 18–46 years, where more than 120 mRNAs and 15 microRNAs demonstrated age-dependent expression levels[47]. We extend this published work by offering the first human platelet RNA-seq dataset comparing younger (age<45 years) and older (age≥65 years) individuals. We have made this dataset available and hope it will serve as a discovery tool for investigators in the field.

Conclusions

In conclusion, human aging is associated with changes in the platelet transcriptome and proteome. GrmA is present and bioactive in human platelets, increases during aging, and regulates inflammatory gene synthesis by monocytes. Alterations in the platelet molecular signature and downstream signaling to monocytes may contribute to dysregulated inflammatory syndromes and adverse outcomes in older adults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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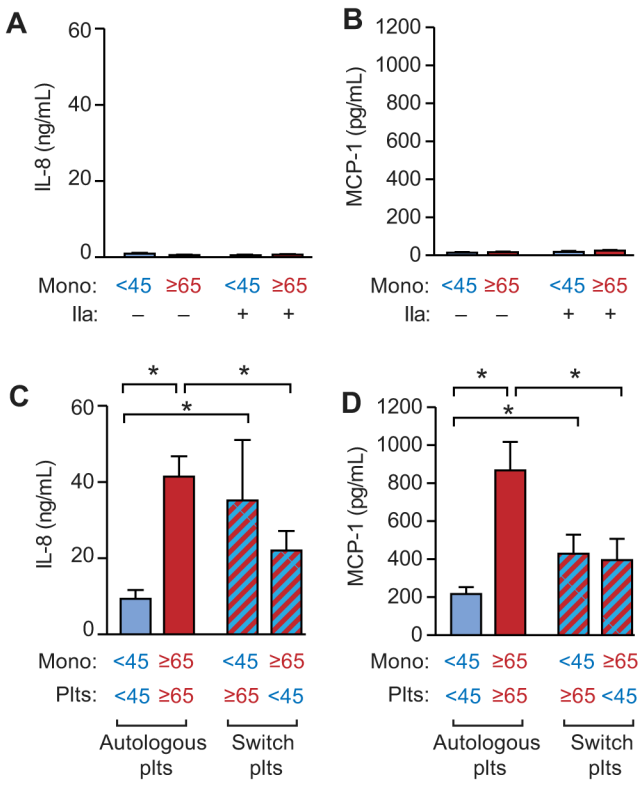

Figure 1. Aged platelets enhance IL-8 and MCP-1 synthesis

Monocytes and platelets were isolated from younger (age<45 years, n=27) or older adults (age≥65 years, n=27). (**Panels A and B**) Monocytes alone were incubated in the presence or absence of thrombin (IIa, 0.1U/mL) for 18 hours. IL-8 and MCP-1 synthesis by monocytes alone did not significantly increase with thrombin and was similar between younger and older adults. (**Panels C and D**) Isolated monocytes were incubated with thrombin activated, autologous platelets or non-autologous (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).

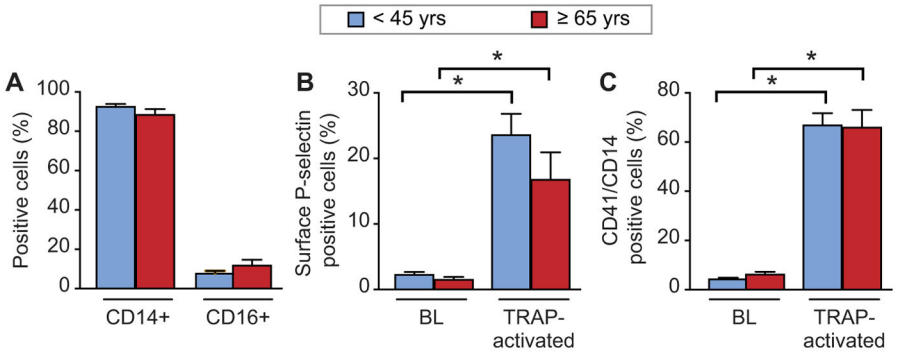

Figure 2. Aging does not alter monocyte CD14 or CD16 surface expression, platelet surface P-selectin expression, or the formation of platelet-monocyte aggregates

Whole blood was drawn from younger (age<45 years) and older subjects (age≥65 years). Blood was stained for surface monocyte markers CD14 or CD16 (**Panel A**, n=6 younger and n=6 older), surface adhesion platelet P-selectin marker CD62p (**Panel B,** n=23 younger and n=13 older), or platelet-monocyte aggregates as measured by double positivity for CD41 and CD14 (**Panel C**, n=26 younger and n=14 older). Expression was adjusted for non-specific staining by using appropriate isotype controls. In **Panels B** and **C**, whole blood was fixed immediately (baseline, BL) or activated with thrombin-receptor activating peptide (TRAP: 5μM for CD41/CD14, 15μM for P-selectin) prior to antibody staining and assessment by flow cytometry (*p<0.05).

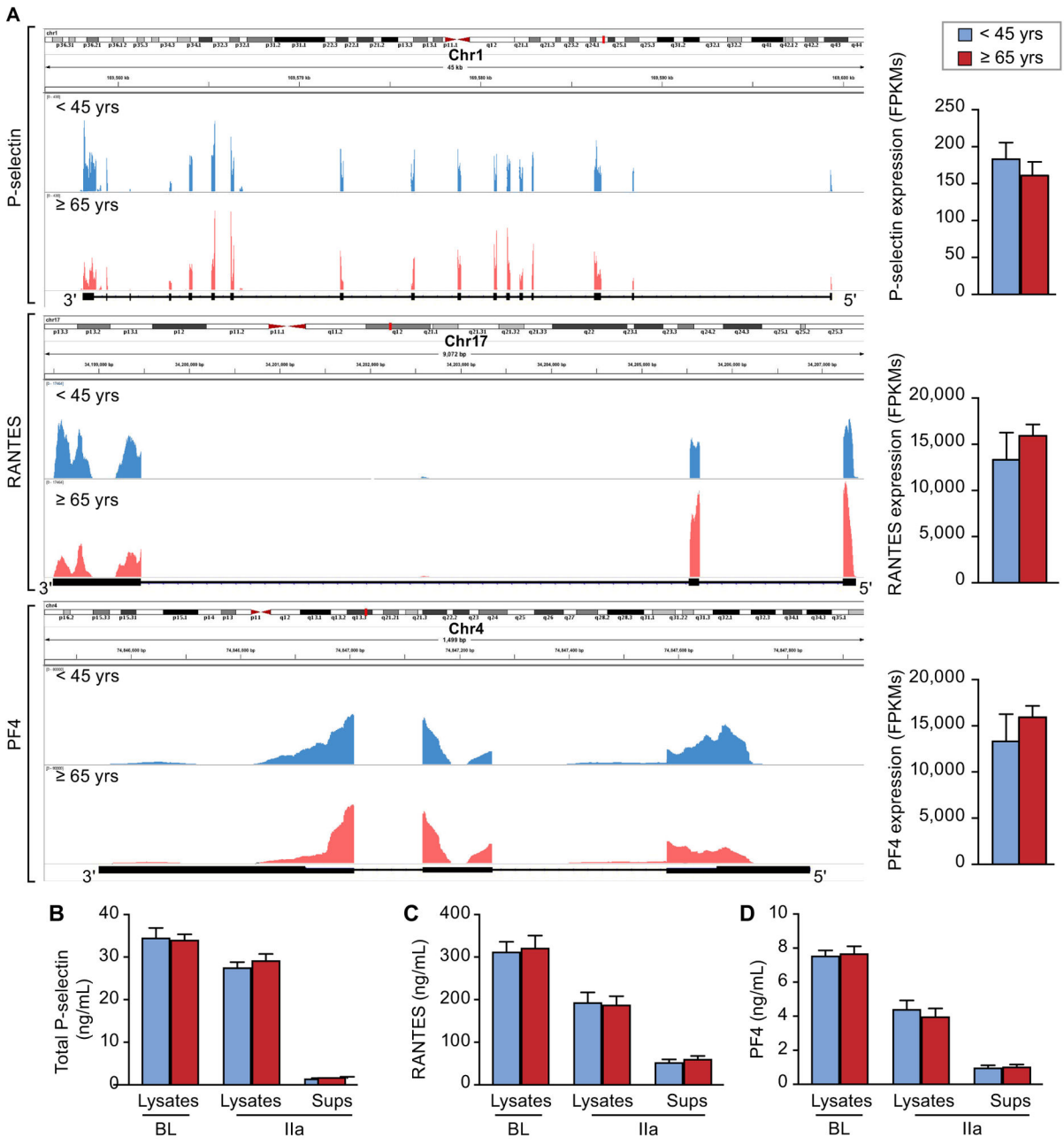

Figure 3. Aging does not alter platelet expression or secretion of the adhesion molecule P-selectin, the signaling molecule RANTES, or the monocyte chemotactic molecule PF4

(Panel A) Platelets were freshly-isolated from younger (age<45 years, n=3) and older subjects (age≥65 years, n=3). Total RNA was isolated from platelets and the platelet transcriptome was assessed by next-generation RNA sequencing (RNA-seq). The total RNA expression of P-selectin, regulated on activation, normal T cell expressed and secreted (RANTES), and platelet factor 4 (PF4) was examined in RNA-sequenced, highly-purified platelet lysates. Shown are representative IGV browser images of each transcript in platelets from a younger (top panels, blue) and older (bottom panels, red) adult with quantified transcript FKPM levels on the right (FPKM: Fragments Per Kilobase of transcript per

Million mapped reads; n=3/group). The y-axis represents the relative expression of each mRNA, with higher peaks indicating increased expression. (**Panels B–D**) To determine the protein expression, platelets were isolated from younger (age<45 years, n=11) and older subjects (age≥65 years, n=11). Platelets were either immediately lysed (baseline, BL) or activated with thrombin (IIa, 0.1U/mL, t=30 minutes). Platelet supernatants were harvested by centrifugation. Total P-selectin (**Panel B**), RANTES (**Panel C**), and PF4 (**Panel D**) protein levels were measured in platelets lysates (for total protein) and in supernatants (Sups) by ELISA.

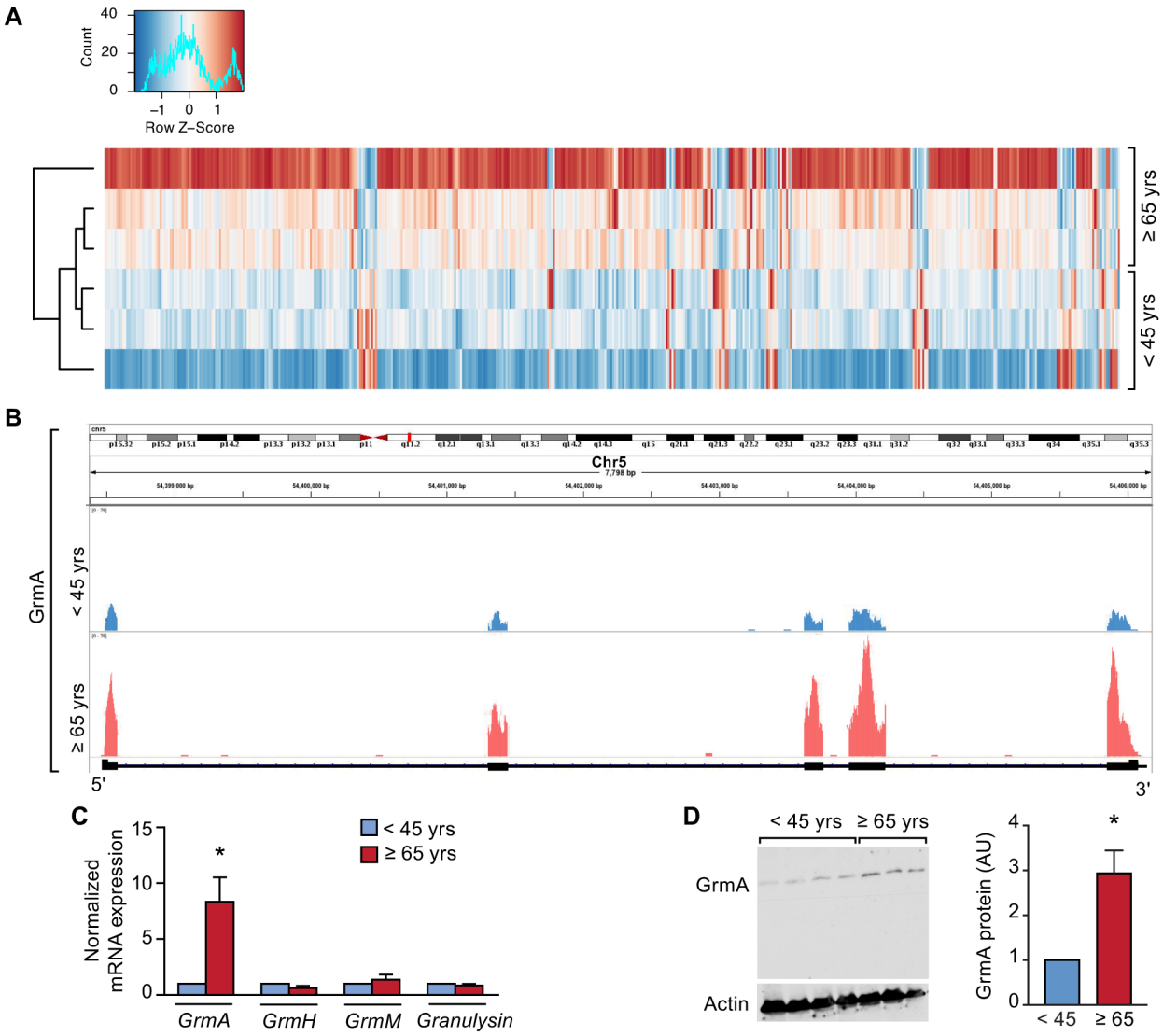

Figure 4. Granzyme A is present in human platelets and its expression increases with aging

(**Panel 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 sequencing (RNA-seq). All transcripts identified by RNA-seq were then filtered to identify only those transcripts that were differentially expressed ($\log_2$ fold-change≥2). The heat map illustrates only those differentially-expressed ($\log_2$ fold-change≥2), upregulated (red) and downregulated (blue) transcripts that were identified in platelets from older adults as compared to younger adults. (**Panel B**) Representative IGV browser images of *granzyme A* (*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). (**Panel C**) *Granzyme A* expression was quantified by qRT-PCR in platelets isolated from older and younger adults. *Granzyme A* expression, but not *granzyme H*

(*GrmH*), *granzyme M* (*GrmM*), or *granulysin* expression, was significantly increased in older adults (*p<0.05 vs. younger). (**Panel D**) Protein expression of GrmA was measured in isolated platelets ($5x10^7$ 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 vs. younger).

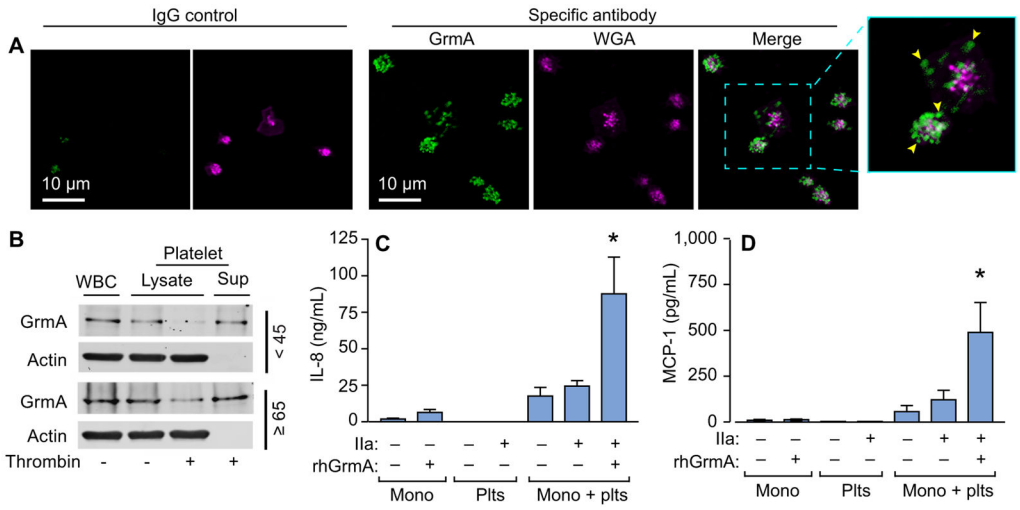

Figure 5. Granzyme A is present in human platelets, secreted upon activation, and induces synthesis of IL-8 and MCP-1

(Panel A) Platelets were isolated from an older adult (age≥65) and stained with an anti-GrmA antibody (GrmA, magenta, right panels) or isotype IgG control (left panels). Platelets were co-stained with wheat germ agglutinin (WGA, green), which stains granules and membranes of platelets. The merged image and inset on the right illustrates the overlay of GrmA (yellow arrows) and WGA. Scale bar is shown on the lower left. Representative of n=3 independent experiments. **(Panel B)** Platelets were isolated from young (age<45) or older adults (age≥65) and either left alone or stimulated with thrombin (IIa, 0.1U/mL; t=30 minutes). The immunoblot shows that GrmA is present at baseline in the platelet lysate and is secreted almost entirely into the platelet supernatant upon stimulation (WBC: white blood cell lysate, positive control). Levels of both intracellular and secreted GrmA were increased in older adults, as compared to younger adults. Actin is shown as a loading control (note that actin is absent from the supernatant, as expected). Representative of n=3 independent experiments. **(Panels C–D)** Platelets are necessary for GrmA-induced synthesis of IL-8 and MCP-1 by monocytes. Human monocytes and platelets were isolated as described in the materials and methods. Monocytes ($2x10^6$ cells/mL, final concentration) were left alone or incubated with recombinant human GrmA (rhGrmA, 100nM final, t=18 hours). Platelets ($2x10^8$ cells/mL, final concentration) were left alone or activated with thrombin (IIa, 0.1U/mL, t=30 minutes). Monocytes and platelets were also incubated together in the absence or presence of recombinant human granzyme A (rhGrmA, 100nM final, t=18 hours). Supernatants were harvested by centrifugation. IL-8 (**Panel C**) and MCP-1 (**Panel D**) levels were measured by ELISA (*p<0.05 vs. all other conditions, n≥3 independent experiments).

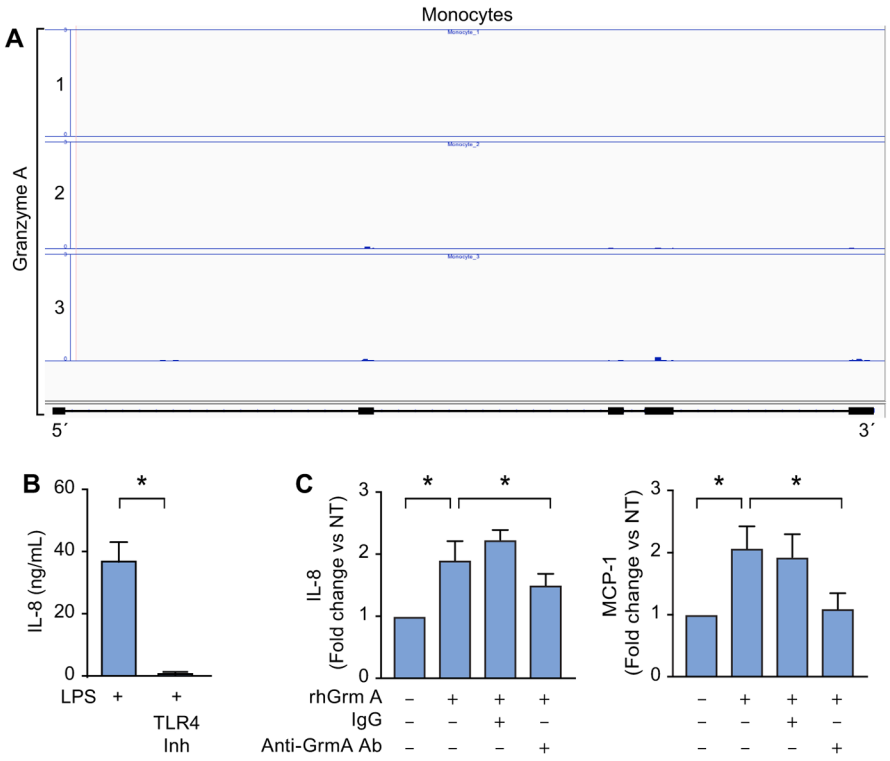

Figure 6. GrmA is not present in resting human monocytes and blocking FcγR does not reduce GrmA-induced IL-8 or MCP-1 synthesis

(Panel A) *Granzyme A* mRNA is not present in resting human monocytes. Human monocytes were isolated as described in the materials and methods. Isolated monocytes were interrogated by next-generation RNA-sequencing. On the bottom, the thick bars on the x-axis illustrate the exons with the 5′ and 3′ ends annotated (left and right, respectively). Shown are IGV browser images from n=3 independent experiments. **(Panel B)** Blocking TLR4 on monocytes prevents IL-8 synthesis. Monocytes were isolated from younger adults. Monocytes were stimulated with LPS (100ng/mL, t=18h) in the presence or absence of a TLR4 inhibitor. IL-8 was measured by ELISA in harvested supernatants (*$p<0.05$, n=3 independent experiments). (**Panel C)** Blocking FcγR does not inhibit GrmA-induced IL-8 or MCP-1 synthesis. Human platelets and monocytes were isolated as described in the materials and methods. Platelets (activated with thrombin, 0.1U/mL) and monocytes were left alone (NT) or stimulated with recombinant human GrmA (100nM). At the same time, either an IgG_1 antibody, which blocks FcγR, or a specific antibody against GrmA (Anti-GrmA) were added to the incubating cells (t=18 hours). IL-8 and MCP-1 were measured by ELISA in harvested supernatants (*$p<0.05$, n=3 independent experiments).

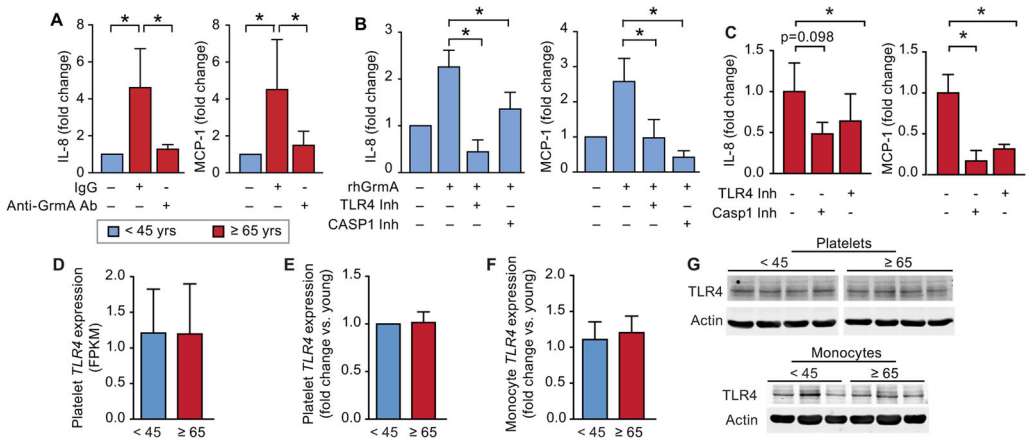

Figure 7. Blocking endogenous GrmA inhibits IL-8 and MCP-1 synthesis in a TLR4 and Caspase-1 dependent mechanism

(**Panel A**) Monocytes ($2x10^6$ cells/mL, final concentration) and platelets ($2x10^8$ cells/mL, final concentration) were isolated from younger (age<45 years, n=5) or older adults (age≥65 years, n=5). Monocytes and platelets were co-incubated together (t=18 hours) together in the presence of thrombin (IIa, 0.1U/mL, t=30 minutes) and an anti-granzyme A blocking antibody (Anti-GrmA) or IgG control antibody (IgG). Supernatants were harvested by centrifugation. IL-8 and MCP-1 protein levels in supernatants were then measured by ELISA. Shown is the fold-change in IL-8 and MCP-1 synthesis in older adults (red) as compared to younger adults (blue) (*p<0.05). (**Panel B**) Monocytes and platelets were isolated from younger adults (age<45 years, n=5). Recombinant, human granzyme A (rhGrmA, 100nM final) was added to the cells in the presence of a TLR4 inhibitor or, separately, a caspase-1 inhibitor. Cells were allowed to incubate together for 18 hours. Supernatants were harvested by centrifugation. IL-8 and MCP-1 protein levels in supernatants were measured by ELISA (*p<0.05). (**Panel C**) Blocking endogenous GrmA in older adults reduces IL-8 and MCP-1 synthesis in a TLR4 and Caspase-1 dependent mechanism. Monocytes ($2x10^6$ cells/mL, final concentration) and platelets ($2x10^8$ cells/mL, final concentration) were isolated from older adults (age≥65 years, n=5). Recombinant, human granzyme A (rhGrmA, 100nM final) was added to the cells in the presence of a TLR4 inhibitor or, separately, a Caspase-1 inhibitor. Platelets and monocytes were allowed to incubate together for 18 hours in the presence of thrombin (0.1U/mL). Supernatants were harvested by centrifugation. IL-8 and MCP-1 protein levels in supernatants were measured by ELISA (*p<0.05). **(Panels D–G)** TLR4 expression on platelets and monocytes does not differ between younger and older subjects. Human platelets and monocytes were isolated from younger (<45 years) and older (≥65 years) as described in the materials and methods. Platelet *TLR4* mRNA expression was examined by RNA-seq (**Panel D,** n=3 subjects per group) and by qRT-PCR (**Panel E,** n=3 subjects per group). **(Panel F)** Monocyte *TLR4* mRNA expression was examined by qRT-PCR (n=3 subjects per group). **(Panel G)** TLR4 protein expression was determined in platelets and monocytes from younger (<45 years) and older (≥65 years) subjects by immunoblot (n=3–4 subjects per group). Actin, shown on the bottom of each blot, was used as a loading control.

Table 1

Characteristics of study cohort

Data represent the mean±SD, unless otherwise indicated.

	Young (n=37)	Old (n=30)	p value
Age, years	27.1±7.2	71.9±6.1	p<0.001
Male Gender, n (%)	17 (46%)	13 (43%)	p=0.85
BMI, kg/m^2	23.7±4.0	28.7±5.5	p<0.001
Aspirin Use, n (%)	1 (3%)	10 (34%)	p<0.05