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Research Article

Megakaryocyte emperipolesis in myeloproliferative neoplasms: Are neutrophils friends or foes?

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Abstract

Megakaryocyte emperipolesis is a biological process in which a cell penetrates and exists as a viable intact cell within another. It is a recognized morphological feature of myeloproliferative neoplasms (MPNs), in which neutrophils can be seen within megakaryocytes in bone marrow smears and sections. We aimed to determine whether neutrophil contents, specifically protein and RNA, are deposited within megakaryocytes due to emperipolesis. Evaluation of hematoxylin and eosin–stained bone marrow showed that 84% of MPN patients (n = 163) had megakaryocyte emperipolesis, most notably in enlarged megakaryocytes and those with pyknotic/condensed nuclei. Morphological assessment and immunohistochemical staining for CD15-neutrophil membrane antigen confirmed that majority of intramegakaryocytic cells were neutrophils, and that emperipolesis was more frequent in myelofibrosis patients and patients with pathologic reticulin. Furthermore, megakaryocytes in MPNs were observed to have intracellular positivity for neutrophil azurophilic granule protein myeloperoxidase (MPO) (n = 21 MPN patients) and specific granule lactoferrin (n = 56). Platelets were also used as a surrogate to establish if neutrophil contents had been transferred into megakaryocytes intracellularly of MPN patients, using mass spectrometry to assess protein and transcriptomic next-generation sequencing to assess messenger RNA (mRNA). A total of 109 neutrophil mRNA transcripts and 20 neutrophil granule proteins were upregulated in platelets of MPN patients compared with control subjects, including cathepsin-G and lactoferrin, with 5.1- and 4.6-fold increase in mRNA and 1.8- and 1.4-fold protein increases, respectively. This suggests that the transfer of neutrophil material occurs during emperipolesis in disease state, which could be a consequence of neutrophil degranulation or apoptosis.

Keywords: emperipolesis, megakaryocyte, myeloproliferative neoplasms, neutrophils, platelets

1. Introduction

Megakaryocyte emperipolesis is a phenomenon in which a hemopoietic cell penetrates, exists as a viable intact cell within, and exits a megakaryocyte.[1] It is recognized to occur in reactive, inherited, and malignant blood disorders (e.g. lymphoma, myeloproliferative neoplasms [MPNs], and myelodysplastic syndromes).[1–8] Emperipolesis is thought to be a physiological response to chemoattractants, resulting in neutrophil adherence to and passage through the megakaryocyte cell membrane.[9] This is initiated by ICAM-1 (intercellular adhesion molecule 1)/ezrin clustering on the megakaryocyte surface, which facilitates interaction with neutrophil β2 integrins.[10] Other interactions, such as CD62P (P-selectin)/PSGL-1 (P-selectin glycoprotein ligand-1) binding, may have a predominant role in emperipolesis occurring under pathological conditions, especially in the context of hematological malignancies.[1,8,11–13] Neutrophils enter the megakaryocyte through a vesicle, which then dissolves, and the neutrophil is free to roam the megakaryocyte cytoplasm and demarcation membrane system until exit.[14,15] In C57BL/6J mice, this process is typically completed within 5 min ("fast emperipolesis"), or can take at least 60 min ("slow emperipolesis").[15]

Although the phenomenon is well described, it remains unclear whether the neutrophils are passive bystanders within the megakaryocyte or if there are biological consequences for the host megakaryocyte. Mouse models suggest the latter, having shown that emperipolized neutrophil cytoplasmic and lipid material can be transferred into megakaryocytes and platelets.[14] In another study, myeloperoxidase (MPO)-positive granules were identified within the megakaryocyte cytoplasm in the vicinity of

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emperipolized neutrophils in a mouse model of myelofibrosis.[3,16] Similar results have been reported in human megakaryocyte emperipolesis, showing the presence of MPO-positive granules to be detectable in the megakaryocyte cytoplasm.[3] The identity of such lipids and proteins transferred from neutrophils and other cells to megakaryocytes and platelets is not known. In gray platelet syndrome (GPS), a bleeding disorder characterized by absence of platelet α-granules, megakaryocyte emperipolesis is a regular event.[6,12,17,18] GPS patients have a loss-of-function mutation in *NBEAL2*, which prevents granule retention in platelets and neutrophils.[17–19] It is thought that emperipolesis may have a role in GPS pathogenesis through the mislocalization of CD62P on the cell surface of megakaryocytes.[12] Platelet RNA sequencing and platelet proteomics of GPS have shown upregulation of neutrophil granules, which was thought to be the result of emperipolesis and transfer of neutrophil content.[18]

MPNs are clonal bone marrow disorders resulting in uncontrolled myeloid proliferation and megakaryocytic atypia. Megakaryocyte emperipolesis is a commonly recognized morphological feature in MPN bone marrow smears and trephine biopsies.[2–4,20–23] It has been described as "internal wandering" of myeloid cells in the cytoplasm of large mature megakaryocytes that are characteristic of MPNs.[22] Electron microscopy studies show that it is present in most patients with MPNs.[3,21,24,25] Neither the mechanism driving emperipolesis in MPNs nor the pathological consequences are well described. In the present study, we have investigated whether there is deposition of neutrophil components (including proteins and RNA) in megakaryocytes and platelets as a consequence of emperipolesis. We first explored megakaryocytes in situ in bone marrow trephines using immunohistochemistry, and then progressed to analyze platelet protein and RNA using mass spectrometry and next-generation sequencing (NGS), respectively.

2. Materials and methods

2.1 Patient recruitment

The project was approved by the Sir Charles Gairdner Osborne Park Health Care Group (2012-094, RGS-1867, RGS-1894, RGS-3807) and the University of Western Australia Human Research Ethics Committees (#RA/4/1/6566, #RA/4/1/9100, #RA/4/1/9009, #2022/ET000097), in accordance with the Declaration of Helsinki. A total of 221 consenting patients with MPNs were recruited, and 42 individuals of similar age and no history of MPNs, reactive, and inherited conditions that may lead to emperipolesis. The makeup of the patient cohorts is shown in Figure S1. Patient characteristics are shown in Table S1.

2.2 Bone marrow pathology

Bone marrow trephine biopsies were fixed, decalcified and processed into paraffin. Hematoxylin and eosin (H&E) and Gordon and Sweet reticulin staining was performed on 3 μm sections (performed by PathWest Laboratory Medicine WA). The morphology of 3 μm sections stained with H&E was reviewed by 2 independent observers (R.J.C. and W.N.E.) in accordance with the 2024 World Health Organization Classification, with a minimum of 30 megakaryocytes analyzed for each specimen.[20,26]

Tissue microarrays of 1.5 mm cores of each bone marrow biopsy were generated using the TMA Master tissue microarray (3DHISTECH). Sections were cut at 3 μm onto charged glass slides (Hurst Scientific). Immunohistochemical single staining was performed using an automated BOND RX Immunostainer (Leica Biosystems) as per Malherbe et al.[27] The primary antibodies used include: CD61 (Clone 2f2; Leica Biosystems), CD15 (Clone Carb-3; Agilent Technologies), MPO (Clone 59A5; Dako), lactoferrin (LTF; Polyclonal; Atlas Antibodies). Double staining was performed using the BOND sequential dual staining protocol for identification of neutrophils (CD15) within megakaryocytes (CD61).[28] A diaminobenzidine substrate BOND Polymer Refine Detection kit (Leica Biosystems) was applied to detect antigen binding for CD15, MPO, and LTF, and BOND Polymer Refine Red Detection kit (Leica Biosystems) was applied for CD61.

R.J.C. and W.N.E. independently reviewed the immunohistochemical staining using an Olympus BX53 light microscope at ×400 magnification (Olympus Life Science Solutions) and imaged using a Pixera Pro600ES digital camera system and Viewfinder application (Pixera). Megakaryocytes were quantified for internalized CD15 or MPO-positive neutrophils and expression of MPO and LTF. Sections below the minimum threshold of 30 megakaryocytes were excluded from quantitative analysis, as per Insuasti-Beltran et al.[26]

2.3 Platelet protein analysis

Peripheral blood (4 mL) from MPN patients and control subjects was collected in CTAD vacutainers (Greiner Bio-One) and processed within 30 min for platelet-rich plasma. Platelet proteins were subsequently isolated and washed as per García.[29] Platelet yield and purity were assessed using a Sysmex XS-1000i Automated Hematology Analyzer as per Collinson et al.[30] Platelet purity was <1 leukocyte per 3.7×10^6 platelets and <1 erythrocyte per 0.1×10^6 platelets, comparable to previous platelet protein studies.[31,32] Platelets were sonicated and lysed, and the protein concentration determined with a bicinchoninic acid assay.[33] Samples were reduced, neutralized, and then digested in trypsin (1:50).[33] Neat solubilized sample was taken to construct a pooled sample.

SWATH-MS (Sequential Window Acquisition of All Theoretical Mass Spectra) analysis of the platelet lysate was conducted as per Bjelosevic et al.[34] IDA (Information Dependent Analysis) and SWATH libraries were constructed with a fraction of the pooled sample. Ion library generation was pooled using the remaining digested samples. IDA libraries were constructed using ProteinPilot (v5.0) (Sciex) using the Paragon algorithm in thorough mode, using 20,294 proteins from the *Homo sapiens* species on UniProt. The unused score cutoff was set to 1.3 (95% confidence for identification) and global protein false discovery rate (FDR) of 1%. The local ion library of 3,147 proteins was constructed from IDAs by merging the 1D-IDA with the 2D-IDAs. Ion library and SWATH data files were imported into PeakView (v2.2) (Sciex). Peptides (maximum 100 peptides per protein) with confidence >99% and FDR <1% (based on chromatographic feature after fragment extraction) were used for quantitation.

Platelet protein subcellular localization was defined using Gene Ontology (GO) terms (https://geneontology.org/). Neutrophil granule localization was determined using Rørvig et al.[35] neutrophil proteome study. Exclusion of neutrophil granules found in platelet granule content was determined using well-established platelet proteome studies.[36–38] The PlateletWeb database (https://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php) was used to assess if proteins had been detected in the platelet proteome previously.[39]

GO enrichment analysis of "cellular component" from significantly differentially expressed proteins between MPN platelets and control subjects was performed using the PANTHER

Overrepresentation Test (PANTHER 19.0; https://geneontology.org/) with *H. sapiens* reference list.[40,41] Statistical significance of mapped pathways was calculated using a Fisher's exact test with FDR correction for adjusted $P < 0.05$. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) based on significantly expressed proteins in platelets between MPN patients and control subjects. "Canonical Pathway Analysis" was stratified as "activated" or "inhibition" based on a z score above −2 and below 2, as per Krämer et al.[42] Reactome overrepresentation analysis (Reactome v88; https://www.reactome.org) was performed on significantly expressed proteins in platelets between MPN patients and control subjects.[43] Statistical significance was determined by a hypergeometric distribution (P value < 0.05) with an adjusted $P < 0.05$ (using the Benjamini-Hochberg adjustment).[43]

2.4 Platelet messenger RNA analysis

Platelets were isolated from EDTA anti-coagulated blood (Greiner Bio-One) within 72 h of collection.[30] Platelets were assessed for purity and prepared for transcriptomic NGS as previously described.[30] In brief, RNA was extracted from platelets using a miRNeasy Mini Kit (QIAGEN) and quantified on a 2100 Bioanalyzer (Agilent Technologies) using an Agilent RNA 6000 Pico Kit. Libraries were prepared using 5 ng of total RNA and an Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) as per Collinson et al.[30]

The Torrent Suite Software v4.4 (Thermo Fisher Scientific) was used to process barcoded reads, perform base calling, align reads to the reference transcriptome (human genome build 19), generate run metrics, and produce gene expression counts. Differential gene expression analyses and count normalization were performed using the DESeq2 package (R/Bioconductor; http://www.bioconductor.org/).[44,45] GO enrichment analysis of the "cellular component" from significantly differentially expressed genes between MPN and control platelets was performed as per protein analysis.

Dysregulated expression of 20 transcripts encoding neutrophilic proteins was selected based on the fold changes in expression and assessed by quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan Gene Expression Array Cards (Thermo Fisher Scientific) (Table S2) as previously described.[44] This included the expression of genes encoding representative proteins from azurophilic granules (*AZU1*, azurocidin 1; *BPI*, bactericidal permeability-increasing protein; *CTSG*, cathepsin G; *DEFA4*, defensin 4; *ELANE*, neutrophil elastase; *MPO*), specific granules (*LTF*; *MMP8*, matrix metalloproteinase-8; *SLPI*, secretory leukocyte peptidase inhibitor), gelatinase granules (*PGLYRP1*, peptidoglycan recognition protein 1), and secretory vesicles (*PRTN3*, proteinase 3). Data were analyzed using the comparative $\Delta\Delta$Ct method.

2.5 Statistical analysis

Statistical significance of age between MPN patients and control subjects was assessed by Mann-Whitney *U* test, whereas sex was assessed by a chi-square test. Comparisons between MPN patients and control subjects were performed with separate chi-square tests for H&E and CD15/CD61 detection of neutrophil emperipolesis, and MPO and LTF immunohistochemical staining. The Mann-Whitney *U* test was applied to compare the mean megakaryocyte emperipolesis per patient (%), and a Kruskal-Wallis one-way analysis of variance (followed by post hoc Dunn's multiple comparisons test [adjusted $P < 0.05$]) was performed to compare between control and individual subtypes, as well as control and reticulin grade <2 and ≥2. SWATH-MS protein peak areas were normalized to the total peak area of the respective sample and subjected to independent *t* test to compare relative protein peak areas. Platelet protein normalized counts of MPN patients and control subjects were analyzed using an independent *t* test. Statistical significance of platelet messenger RNA (mRNA) fold change was defined using Wald test (*P* value), followed by a Benjamini-Hochberg *P* value adjustment < 0.05 (DESeq2).[45] For qRT-PCR comparison, statistical significance between MPN patients and control subjects was determined using a Welch's *t* test with a *P* value. Statistical significance was set at a *P* value of <0.05.

3. Results

3.1 Bone marrow immunohistochemistry

Megakaryocyte emperipolesis, defined as a cell fully internalized and intact within a megakaryocyte, was present in 84% of MPN (n = 163) patients and 30% of control subjects (n = 27) on H&E-stained sections ($P = 0.011$). CD61/CD15 dual staining revealed that the majority of internalized cells within the megakaryocyte cytoplasm of CD61-positive cells were CD15 positive (Fig. 1) and not segments of sectioned megakaryocyte nuclei or other hemopoietic cells. The morphology combined with the CD15 positivity established these as neutrophils. In 63% (n = 102 of 163) of MPN patients and 22% (n = 6 of 27) of control subjects there was at least 1 megakaryocyte with an intracytoplasmic CD15-positive neutrophil ($P = 0.022$). Morphological assessment showed that the CD15-positive neutrophils were preferentially within large or "giant" megakaryocytes with hyperlobated nuclei (Fig. 1A, D, E). On rare occasions, emperipolesis occurred in smaller megakaryocytes with pyknotic angulated nuclei with hyperchromatic chromatin stain (Fig. 1B, C, E).

The mean percent (%) megakaryocytes emperipolized was significantly greater in bone marrow sections of MPN ($\bar{x} = 8.3\%$, range = 0% to 43.9%) than the control biopsies ($\bar{x} = 2.6\%$, range = 0% to 36.7%) ($P = 0.001$) (Fig. 2A). This was higher in myelofibrosis (MF) (n = 63; $\bar{x} = 11.62\%$, range = 0% to 42.50%) patients than in essential thrombocythemia (ET) (n = 68; $\bar{x} = 6.57\%$, range = 0% to 43.90%) (adjusted $P = 0.006$) and polycythemia vera (PV) (n = 32; $\bar{x} = 5.58\%$, range = 0% to 34.69%) (adjusted $P = 0.003$) patients. When categorized based on reticulin grade, the mean % megakaryocytes emperipolized was increased in marrow samples with reticulin grade ≥2 (n = 70; $\bar{x} = 12.38\%$, range = 2.18% to 42.50%) compared with grade <2 (n = 93; $\bar{x} = 5.27\%$, range = 0% to 43.90%) (adjusted $P < 0.0001$) and control samples (adjusted $P < 0.0001$) (Fig. 2C). In contrast, reticulin grade <2 was not significantly increased compared with control samples (adjusted $P = 0.222$).

We then investigated whether there was evidence of neutrophil-specific components within the megakaryocyte. For this we assessed MPO and LTF, components of azurophilic and specific neutrophil granules, respectively. In 21 (20.6%) of 102 MPN bone marrows with neutrophil emperipolesis, 1% to 30% of megakaryocytes showed intracellular MPO positivity ($P = 0.0148$) (Fig. 2D), and 56 (54.9%) of 102 for LTF (1% to 59% of megakaryocytes; $P < 0.0001$) (Fig. 2E). In all MPN cases with MPO-positive megakaryocytes, LTF was also positive, and there were morphologically identifiable emperipolized neutrophils (Fig. 3; Fig. S2). These findings differed from the control marrows, in which there was no megakaryocytic MPO or LTF staining, whereas granulocyte progenitors and neutrophils were positive for both (Fig. 3).

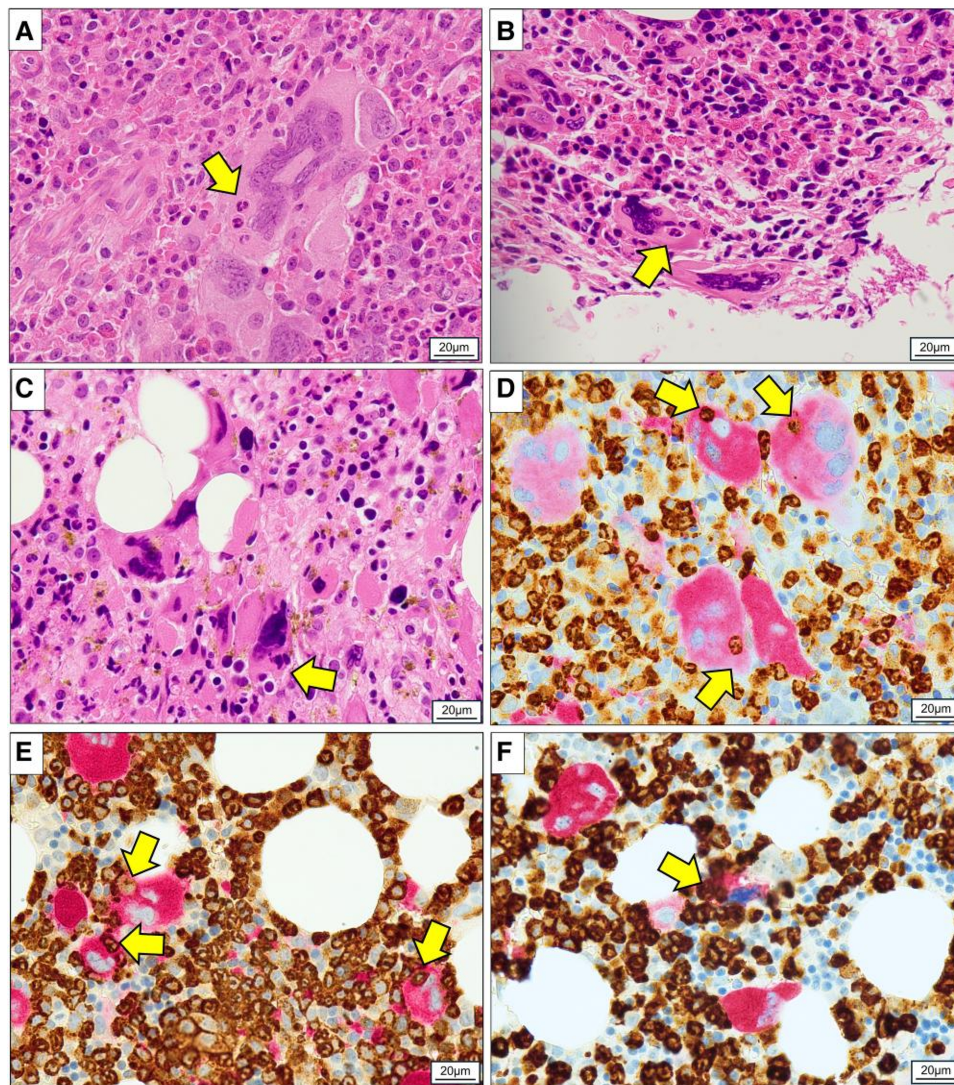

Fig. 1. Bone marrow trephine sections of MPN patients. (A) H&E staining showing emperipolesis in a case of essential thrombocythemia. (B, C) H&E staining showing emperipolesis in 2 cases of myelofibrosis. (D–F) CD61-positive megakaryocytes (Fast red; red) with CD15-positive neutrophil emperipolesis (diaminobenzidine [DAB]; brown) in cases of MPN. Yellow arrows show emperipolesis (×400).

3.2 Platelet protein analysis

Having determined that neutrophil-unique components from both azurophilic and specific granules were present in megakaryocytes, we then assessed platelets for neutrophil proteins. From 27 MPN patient samples and 3 control samples, a total of 2,499 platelet proteins were detected by SWATH-MS analysis (Fig. S3A). The expression of 482 proteins (402 upregulated, 80 downregulated) were significantly different between MPN patients and control subjects. Of these, there were 20 proteins unique to neutrophil granules or granule membranes that were upregulated; none have been reported to be present in platelets (GO annotations), and 6 have never been detected in the platelet proteome according to the PlateletWeb database (Table 1).[36–39] Of those neutrophil granule elements upregulated in MPNs, several have known functional interactions with the platelet/megakaryocyte proteome (Table S3).

Pathway analysis was performed on the significantly expressed proteins to provide further insight on any neutrophil-associated functional changes that might be occurring in the megakaryocyte-platelet lineage. GO cellular component enrichment analysis was performed on the significantly differentially expressed proteins, in which 192 mapped pathways were enriched (adjusted $P < 0.05$), including secretory vesicle (GO:0099503; 3.74-fold, adjusted $P = 6.48 \times 10^{-27}$), secretory granule (GO:0030141; 6.92-fold, adjusted $P = 4.96 \times 10^{-26}$), ficolin-1-rich granule (GO:0101002; 7.06-fold, adjusted $P = 2.80 \times 10^{-15}$), and azurophil granule (GO:0042582; 5.37-fold, adjusted $P = 1.06 \times 10^{-7}$) (Fig. S3B). IPA and Reactome pathway analysis illustrated that “neutrophil degranulation” was the most statistically enriched pathway (IPA z score = 5.695 and P value = 3.45×10^{-28}; Reactome adjusted $P = 2.16 \times 10^{-9}$) (Fig. S4 and Table S4).[42,43]

In the platelet proteomics analysis, there was no difference in expression of lymphocyte-associated proteins between MPN-derived and control platelets, including AGK (acylglycerol kinase) (1.16-fold, $P = 0.205$), LYN (LYN proto-oncogene Src family tyrosine kinase) (0.93-fold, $P = 0.492$) and SYK (spleen associated tyrosine kinase) (0.51-fold, $P = 0.281$) (Table S5).

3.3 Platelet RNA analysis

Having established that neutrophil protein contents appeared to have been present within megakaryocytes in MPNs, we performed

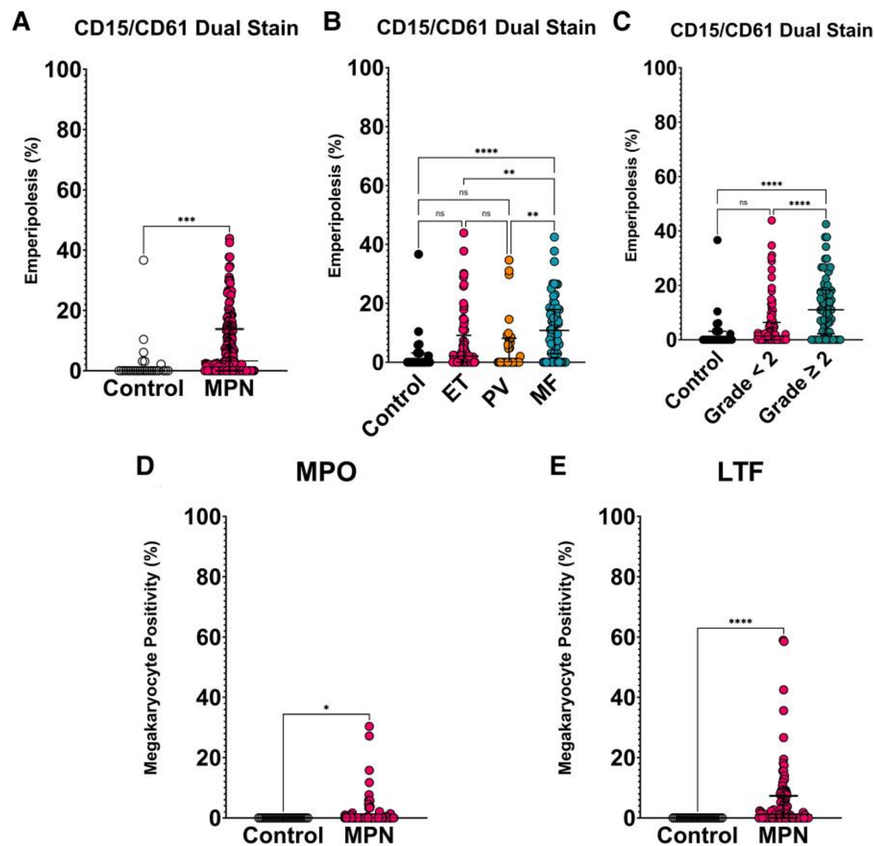

Fig. 2. Immunohistochemical staining of bone marrow trephine sections comparing between MPN patients and control subjects. (A) Mean percentage (%) of megakaryocyte (CD61-positive) CD15-positive emperipolesis per patient (Mann-Whitney *U* test [$P < 0.05$]). (B) Mean megakaryocyte emperipolesis comparing control and MPN subtypes (Kruskal-Wallis 1-way analysis of variance followed by post hoc Dunn's multiple comparisons test [adjusted $P < 0.05$]). (C) Mean megakaryocyte emperipolesis comparing the control sample, reticulin grade <2, and grade ≥2 (Kruskal–Wallis 1-way analysis of variance/Dunn's multiple comparisons test). (D, E) Mean megakaryocyte positivity of MPO and LTF (Mann-Whitney *U* test). Data shown as median ± interquartile range. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. ns, not significant.

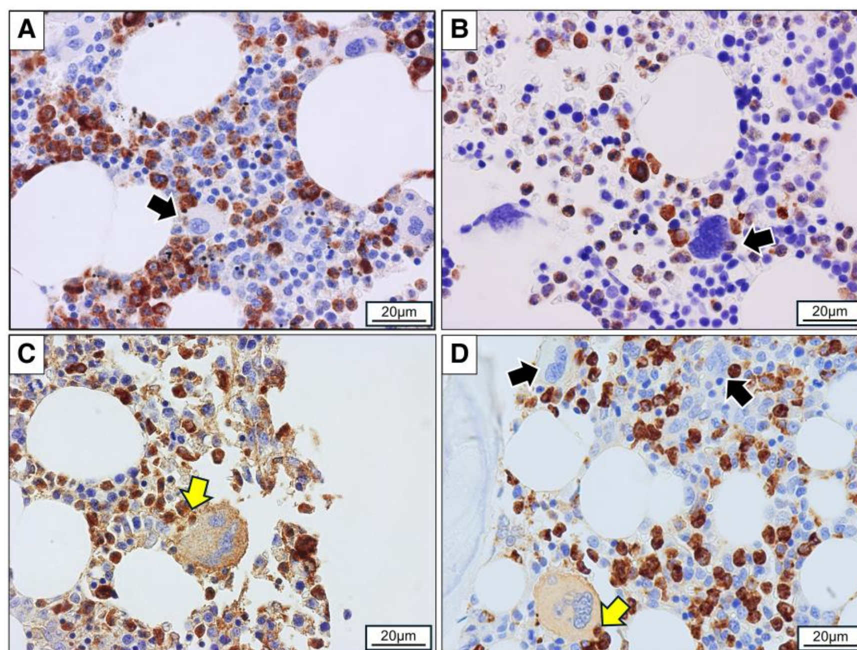

Fig. 3. Immunohistochemical staining of bone marrow trephine sections: (A) MPO-stained control sample; (B) LTF-stained control sample; (C) MPO-stained MPN sample; and (D) LTF-stained MPN sample (diaminobenzidine [DAB], brown). MPO and LTF staining in control samples shows positive emperipolesis staining of neutrophils, but megakaryocytes are negative, whereas MPN marrows show positive intracellular positivity in megakaryocytes. The yellow arrows indicate megakaryocytes containing emperipolized neutrophils within a positive megakaryocyte. The black arrows indicate negative megakaryocytes with emperipolesis. Hematoxylin counterstain (×400).

Table 1. Upregulated expression of 20 neutrophil-specific proteins in platelets in MPN compared with control subjects.

Granule Localization[a]	Protein Name (Gene ID)	Fold Change	P Value
Azurophilic granule membrane	Inositol 1,4,5-triphosphate receptor associated 2 (LRMP)[b]	3.43	4.36×10^{-5}
	Copine 3 (CPNE3)	1.58	0.002
Azurophilic granule	Cathepsin G (CTSG)[b]	1.77	0.029
	Gamma-glutamyl hydrolase (GGH)	1.50	0.014
Specific granule	Lactoferrin (LTF)	1.40	0.018
	Endoplasmic reticulum protein 44 (ERP44)	1.19	0.044
Gelatinase granule membrane	Ectonucleotide pyrophosphatase/phosphodiesterase 4 (ENPP4)[b,c]	1.36	0.026
Gelatinase granule	Lamin B receptor (LBR)	1.61	0.020
	Cathepsin B (CTSB)[b,c]	3.30	4.05×10^{-5}
	Acylaminoacyl-peptide hydrolase (APEH)[c]	1.28	0.025
	PDGFA associated protein 1 (PDAP1)[c]	1.27	0.012
Secretory vesicles	RAB4B—member RAS oncogene family (RAB4B)	1.69	0.001
	Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	1.62	0.026
	Torsin family 1 member A (TOR1A)[b]	1.51	0.027
Shared neutrophil granules	Cystatin B (CSTB)	2.83	0.001
	Eukaryotic translation elongation factor 2 (EEF2)	2.13	0.002
	Spectrin alpha, nonerythrocytic 1 (SPTAN1)[b]	1.48	0.044
	Glia maturation factor gamma (GMFG)	1.27	0.009
	NME/NM23 nucleoside diphosphate kinase 2 (NME2)	1.26	0.006
	Phosphoglycerate mutase 1 (PGAM1)	1.19	0.032

[a]Granule where neutrophil protein is most abundant.[35]
[b]Proteins not previously detected in platelet proteome.[39]
[c]Also found in lesser known neutrophil granule "ficolin-1-rich granule."[46]

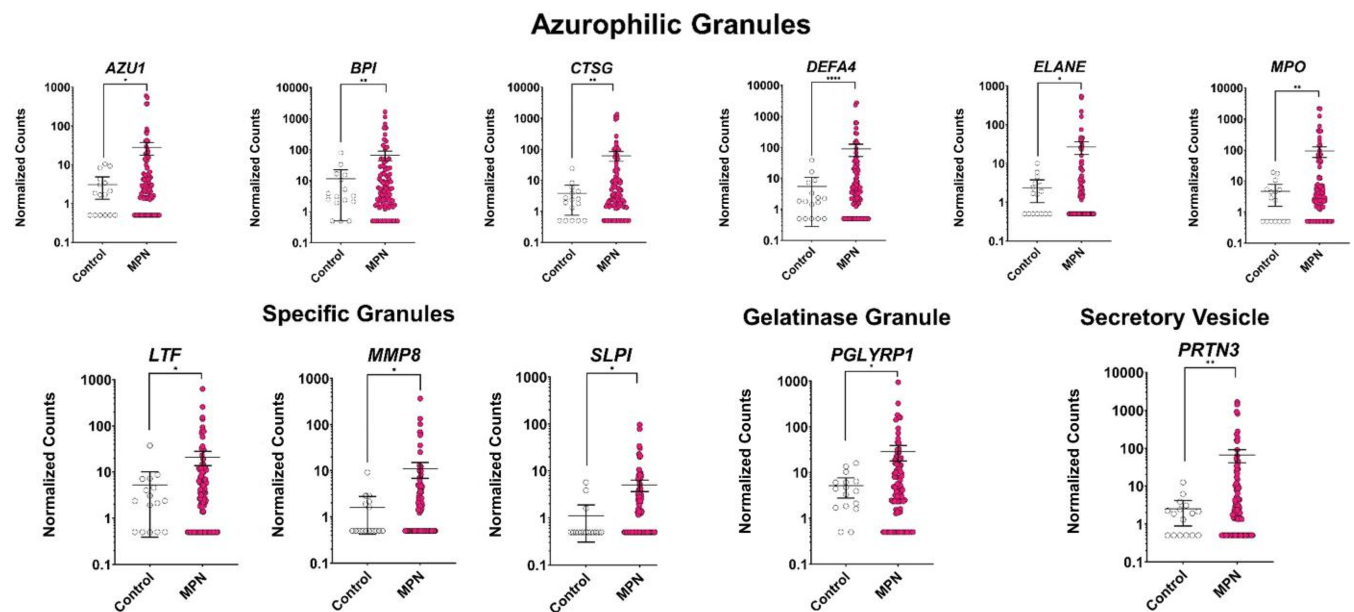

Fig. 4. Increased expression of genes encoding neutrophil azurophilic, specific, and gelatinase granule and secretory vesicle proteins in platelets in MPN patients compared with control subjects. Data shown as mean ± 95% confidence interval. *Adjusted $P < 0.05$, **Adjusted $P < 0.01$, ****$P < 0.0001$.

transcriptomic NGS of platelets from 97 MPN patients and 15 control subjects to assess for neutrophil-associated genes (Fig. S1C).[30] An average of 10.1×10^6 mapped reads at 95.6% valid reads were obtained. A total of 4,610 genes were significantly expressed between MPN patients and control subjects (2,729 upregulated, 1,881 downregulated) (Fig. S2C). GO cellular component enrichment analysis was performed on the significantly differentially expressed genes and identified 415 pathways that were enriched (adjusted $P < 0.05$), including secretory granule (GO:0030141; 1.40-fold, adjusted $P = 1.90 \times 10^{-8}$), azurophil granule (GO:0042582; 2.04-fold, adjusted $P = 2.67 \times 10^{-8}$), secretory vesicle (GO:0099503; 1.33-fold, adjusted $P = 7.21 \times 10^{-7}$), and ficolin-1-rich granule (GO:0101002; 1.67-fold; adjusted $P = 1.75 \times 10^{-4}$) (Fig. S2D).

A total of 131 neutrophil-associated genes, including genes encoding proteins of each neutrophil granule type, were differentially expressed (109 upregulated and 22 downregulated) in MPN. Key changes were seen in genes encoding proteins from azurophilic granules, including *CTSG* (5.1-fold increase, adjusted $P = 0.004$), *CEACAM6* (14.74-fold, adjusted $P = 6.84 \times 10^{-5}$), and *DEFA4* (12.74-fold, adjusted $P = 6.74 \times 10^{-5}$). The expression of *LTF* (specific granules) was also increased by 4.6-fold (adjusted $P = 0.011$). Genes encoding for secretory vesicle proteins, such as *CPA3* (29.92-fold, adjusted $P = 4.75 \times 10^{-12}$) and *SYT9* (22.58-fold, adjusted $P = 4.08 \times 10^{-7}$) were also increased. Details of upregulated mRNA for proteins in azurophilic (*AZU1*, *BPI*, *CTSG*, *DEFA4*, *ELANE*, and *MPO*), specific (*LTF*, *MMP8*, and *SLPI*), and gelatinase granules (*PGLYRP1*) as well as in secretory vesicles (*PRTN3*) compared with control subjects are shown in Fig. 4. In MPNs, lymphocyte-specific genes were either downregulated or showed no difference from control subjects. Examples include *CD4*

(T-lymphocyte surface glycoprotein *CD4*) (1.56-fold, adjusted $P = 0.073$), *CD19* (B-lymphocyte antigen *CD19*) (0.51-fold, adjusted P = not applicable), and *GMZA* (Granzyme A) (0.53-fold, adjusted $P = 0.046$) (Table S5).

qRT-PCR was performed on platelets to verify the overexpression of genes encoding proteins abundant in neutrophil granules, from 54 MPN samples and 12 control subjects. The most significant changes were found in *MPO* (61.18-fold, $P = 0.007$), *SLPI* (3.9-fold, $P = 0.007$), and *PRTN3* (64.38-fold $P = 0.013$).

4. Discussion

Megakaryocyte emperipolesis is a feature of MPNs in which neutrophils were thought to "wander" within the megakaryocyte and be preserved from the bone marrow microenvironment.[47] The data presented suggests that megakaryocytes have acquired neutrophil granule contents (i.e. MPO and LTF) in MPN patients, thereby suggesting that there has been interaction between the neutrophil and megakaryocyte. This is further supported by upregulated expression of neutrophil-unique proteins and mRNA within platelets. As these changes were not seen in control samples, it is inferred that there has been exposure of neutrophil contents to cells of the megakaryocyte-platelet pathway in MPNs. We hypothesize that this exchange of neutrophilic material has occurred as a consequence of emperipolesis with active exchange of cellular material.

Morphological review of bone marrow sections demonstrated that emperipolesis occurred preferentially in large or "giant" megakaryocytes showing hyperlobated nuclei, and in smaller megakaryocytes with contracted and pyknotic nuclei. These findings support results from 2 previous studies that analyzed murine models. First, a C57BL/6J mouse model of emperipolesis showed greater frequency and efficiency of emperipolesis in mature and larger megakaryocytes.[15] Second, the $\text{Gata1}_{\text{low}}$ mouse model that recapitulates myelofibrotic progression exhibited increased emperipolesis of hypolobated and para-apoptotic megakaryocytes.[48] Megakaryocyte hyperplasia and excess cytoplasmic maturation found in MPN patients are instigated by the increase of JAK-STAT signaling.[27,49–52] How neutrophils traffic into these larger or smaller megakaryocytes to begin with is unclear. Theories as to why neutrophils tend to be emperipolized in larger polyploid megakaryocytes include taking sanctuary inside the megakaryocyte from unstable marrow microenvironment, increased chemoattraction though JAK-STAT inflammatory signaling, reduced energy output required for the neutrophil to move through instead of around the larger megakaryocyte, or hypolobated megakaryocytes are thought to increase cell-surface expression of P-selectin, which attracts the emperipolesis interaction.[1,14,53]

Our results have shown that neutrophil-unique membrane and granule proteins are present within MPN megakaryocytes and platelets. This suggests that neutrophils may not be simply "wandering" through and leaving intact, but instead may be losing cellular contents within the megakaryocyte milieu. This may be an active process whereby neutrophils have secreted granule and cytoplasmic contents into the megakaryocyte host. Alternatively, it may be a consequence of neutrophil degradation from cytolytic damage occurring within the megakaryocyte. Evidence in MPN patients and mouse models has shown that MPO-positive granules are localized within the megakaryocyte cytoplasm, near emperipolized neutrophils.[3,16] Studies in C57Bl/6, CD45.1 B6, mT/mG, and Tg-FcγRIIA mice have also shown that intracellular neutrophils leave lipid and cytoplasmic content in megakaryocytes.[14] This infers that emperipolesis is not "passive" in MPNs, but rather leads to loss of neutrophilic cellular constituents into the megakaryocytes.

As previously mentioned, emperipolesis is not restricted to MPNs, and increased rates have been reported in other inherited and acquired conditions. Studies of GPS, in which megakaryocyte emperipolesis is common, have demonstrated upregulation of neutrophil granule contents and RNA from patient-isolated platelets.[18] In both GPS and MPNs, the question arises as to whether the identification of neutrophil species in megakaryocytes and platelets is due to the number of emperipolesis events, or whether there is a defect in the megakaryocyte or neutrophil.[16,18] In MPNs, neutrophils are derived from the clonally defective progenitor cell and are known to be activated with dysregulated apoptotic machinery.[54–57] This may be enhanced during emperipolesis, whereby the abnormal intracellular megakaryocytic milieu may stimulate apoptotic cell death or neutrophil extracellular trap formation.[56,57] Frequent neutrophil NETosis and apoptosis is a feature of MPNs and may potentially drive inflammatory response during disease pathogenesis.[56,58] This was reported in a MPN murine model, in which emperipolesis was associated with para-apoptotic morphology for both the megakaryocyte and the neutrophil.[3,16] Megakaryocyte-derived cytokines, such as interleukin-6, platelet factor 4, and transforming growth factor β, are enriched in MPNs and may trigger neutrophil degranulation, but it is likely dependent on the cytokine concentration intracellularly.[59–63] Our pathway analysis data of differentially expressed proteins in platelets (Fig. S2 and Table S4) showed that "neutrophil degranulation" was the most statistically enriched pathway and therefore indicates that neutrophil degranulation may be at play.[64–66]

There are multiple potential pathological consequences of neutrophil degranulation within megakaryocytes. First, neutrophilic granules may be toxic to megakaryocytes, leading to apoptotic death.[1] This is could explain why megakaryocytes shrink in size and show nuclear pyknosis (as seen in Fig. 1B, C, F), a defining characteristic of MF megakaryocytes with emperipolesis.[3,16,27,67] Furthermore, several of the neutrophil granule elements upregulated in MPN emperipolesis have well-described functional interactions with the platelet/megakaryocyte proteome, particularly with pathways associated with megakaryocyte apoptosis, platelet production, and activation.[68–72] This builds on previous studies that have illustrated a crosstalk between neutrophils and platelets and implies that emperipolesis may facilitate a physiological response to blood loss and injury.[14,73–76]

Second, neutrophil granule proteins are known to be toxic to and can destroy megakaryocyte α-granules, a feature identified at an ultrastructural level in MPNs and also in GPS.[16] Studies in mice have shown that when emperipolesis is blocked, the release of granule contents into the bone marrow microenvironment is reduced, further supporting a role for emperipolesis in megakaryocyte α-granule dysfunction.[9,11] In these studies, the depletion of emperipolesis coincided with the reduction of pathologic reticulin. Our results in human bone marrow samples support these mouse findings as an increase in emperipolesis frequency was found to be correlated with increased reticulin (i.e. World Health Organization grade ≥ 2).[9,11] A recent histological study has recommended that incorporating emperipolesis assessment could improve early diagnosis of MF.[2] Together these data indicate that there is a correlation between emperipolesis and reticulin grade.

From a technical perspective, the 3 μm thickness of bone marrow trephines relative to the size of megakaryocytes (50–200 μm) was a limitation of this study. Furthermore, the difficulty of extracting megakaryocytes from aspirate samples (size and rarity) necessitated the studying of platelets, the cytoplasmic progeny of megakaryocytes, as a surrogate. Using this strategy, we were able to quantify intracellular neutrophil–derived proteins and mRNA.[30,44,77] A restriction of the platelet proteomic approach used was that MPO, the most prevalent neutrophil granule protein, was not detected above the threshold. Other granule proteins that are typically at a lower concentration within normal neutrophils in circulation were detectable (e.g. LBR and CTSB).[78–81] The half-life of MPO in plasma is also shorter than detectable protein LTF, particularly in MPN patients, but it is not known whether MPO is less stable within an intracellular component.[82–84] The selective presence of neutrophil and not lymphocyte-associated mRNA infers that emperipolesis is the most likely source of the neutrophil proteins and mRNAs detected in platelets. However, we cannot exclude that either megakaryocyte or platelet endocytosis may be an alternate source of these proteins and mRNAs.[85–91]

In summary, we have utilized several methodologies which infer that neutrophils are leaving behind cellular components, including azurophilic (MPO) and specific (LTF) granule contents and mRNA, during emperipolesis in MPNs. This suggest that emperipolesis is not merely a "passive" event, as suggested in the initial descriptions of this biological process. Instead, there appears to be an interchange of neutrophil proteins and RNA into the host megakaryocyte. The mechanism by which neutrophils transit into megakaryocytes and then deposit their potentially damaging contents requires further study. Regardless, we believe that in the context of MPNs, neutrophils are likely "foes," and not "friends," to their host, the megakaryocyte.

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Author contributions

R.J.C., B.B.G., M.D.L., K.A.F., J.H.C., W.H.O., and W.N.E. designed the research. R.J.C., B.B.G., and M.D.L. analyzed the data. B.M., L.W., and D.B. isolated platelets. Z.Y.N., H.C., J.A.J.M., R.H., M.F.L., and M.H.S. recruited patients and analyzed the clinical data. H.C., R.J.C., W.N.E., and K.A.F. reviewed and analyzed the bone marrow trephines. R.J.C. performed the pathway analysis. All authors contributed to drafting the paper and had final approval of the submitted and published versions.

Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

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Conflicts of interest. The authors declare that they have no competing interests.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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