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Intramedullary megakaryocytes internalize released platelet factor 4 (PF4) and store it in alpha granules

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Summary

Background—Megakaryocytes express and store platelet factor 4 (PF4) in alpha-granules. In vivo, PF4 is a clinically-relevant, negative regulator of megakaryopoiesis and hematopoietic stem cell (HSC) replication. These findings would suggest a regulated source of free intramedullary PF4.

Objectives—Define the source of free intramedullary PF4 and its intramedullary life cycle.

Methods—We interrogated both murine and human bone marrow-derived cells during megakaryopoiesis in vitro by confocal microscopy and ELISA. Using immunohistochemistry, we examined in vivo free PF4 in murine bone marrow before and after radiation injury and in the setting of megakaryocytopenia and thrombocytopenia.

Results—Exogenously added human PF4 is internalized by murine megakaryocytes. Human megakaryocytes similarly take up murine PF4, but not the related chemokine, platelet basic protein. Confocal microscopy shows that internalized PF4 co-localizes with endogenous PF4 in

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Authorship

M. P. Lambert developed technologies and carried out studies, analyzed the data, and was the primary author of the manuscript. R. Meng and D. C. Harper performed the confocal and immunofluorescence microscopy experiments and data analysis. R. Meng wrote sections of the manuscript. L. Xiao performed and analyzed the tissue culture and flow cytometry experiments, and revised the manuscript. M. S. Marks provided guidance for the microscopy experiments and revised the manuscript. A. M. Kowalska developed and characterized the *Pbp*^{-/-} and hPBP⁺ mice and revised the manuscript. M. Poncz provided overall scientific guidance and manuscript preparation.

Disclosure

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alpha-granules and is available for release upon thrombin stimulation. Immunohistochemistry shows free PF4 in the marrow – but not another alpha-granule protein, von willebrand factor. Free PF4 increases with radiation injury and decreases with megakaryocytopenia. Consistent with the known role of low-density lipoprotein receptor related protein 1 (LRP1) in the negative paracrine effect of PF4 on megakaryopoiesis, PF4 internalization is at least partially LRP1-dependent.

Conclusions—Thus PF4 has a complex intramedullary life cycle with important implications in megakaryopoiesis and HSC replication not seen with other tested alpha-granule proteins.

Keywords

megakaryocytes; platelet factor 4; thrombocytopenia; radiation injury; diphtheria toxin; Low Density Lipoprotein Receptor-Related Protein-1

Introduction

Mature megakaryocytes release platelets, which play a critical role in a number of biological processes including megakaryopoiesis and hematopoiesis.(1, 2) As part of its multiple roles, platelets store and release their granular content at sites of platelet activation.(3) There are three distinct classes of platelet granules – alpha-granules, dense granules and lysosomes – with most platelet-specific proteins stored in alpha-granules.(4) Many of these platelet proteins are synthesized by megakaryocytes and traverse the secretory pathway before storage.(5, 6) Other alpha-granule proteins are synthesized elsewhere and internalized into megakaryocytes or platelets by endocytosis(7, 8) or pinocytosis(9). These granule proteins are widely implicated in platelet biology and pathobiology.(10–12)

One of the most abundant alpha-granule proteins on a molar basis is platelet factor 4 (PF4, CXCL4), a platelet specific, C-X-C family chemokine.(13) PF4 expression has been noted in other hematopoietic lines(14), but the RNA levels are 2 or more orders of magnitude less abundant expression than in megakaryocytes(15) where PF4 is the third most abundant mRNA and the protein makes up 3% of granular content(16). We and others have shown have shown that PF4 has an important intramedullary function in regulating the number of megakaryocytes: platelet PF4 levels are inversely related to steady-state platelet counts(17, 18); the degree of both chemo- and radiation-induced thrombocytopenia in murine models is inversely related to PF4; and the level of PF4 expression in platelets inversely correlates with the need for transfusion after chemotherapy in patients(19). This negative paracrine effect of PF4 on megakaryocytes is mediated by low-density lipoprotein receptor-related protein 1 (LRP1, CD91).(20) Bruns et al., showed that PF4 also regulates hematopoietic stem cell (HSC) replication, but the underlying mechanism is unknown(15).

If intramedullary PF4 levels affect HSC and megakaryocyte replication, what is the source of PF4 and how is its level regulated? We now demonstrate that PF4 is released from megakaryocytes – and not platelets – within the marrow space. This release increases following radiation injury. Significant levels of this free PF4 can then be internalized back into megakaryocytes via LRP1 at levels approaching that of endogenous PF4 and stored in the same alpha granules. This reuptake PF4 is then capable of being released again (functionally recycled). These features are unusual to PF4 and are not seen for other alpha-

granule proteins tested. We thus provide new insights into where intramedullary PF4 comes from and how it is regulated. Whether this complex biology is unique to PF4 because of its intramedullary roles or is seen with other alpha-granule proteins needs further testing.

Methods

Murine lines studied

Several previously-described lines of mice with different levels of PF4 were studied, and include homozygous *Pf4* knockout mice (*Pf4*^{-/-})(21) and mice transgenic for human (h) PF4-expression specifically in megakaryocytes (hPF4⁺)(22). Mice that express an inducible diphtheria toxin receptor (iDTR) during megakaryopoiesis were created by crossing *Pf4*-Cre mice(23) with iDTR homozygous mice(24) to generate mice that were either positive (*Pf4*-Cre⁺/iDTR^{+/+}, termed iDTR⁺) or negative (*Pf4*-Cre⁻/iDTR^{+/+}, termed iDTR⁻) as described¹⁵. In these animals, treatment with diphtheria toxin (DT) results in marked megakaryocytopenia. Finally, homozygous PBP knockout mice (*Pbp*^{-/-}) were studied. *Pbp*^{-/-} mice have all three exons of *Cxcl7* replaced by a neomycin-resistant cDNA⁽²²⁾ (Supplement Figures 1A–B) and have no detectable mouse (m) PBP RNA or protein in circulating platelets (Supplement Figures 1C–D). Transgenic hPBP⁺ mice contain a 6.2 kb fragment of the human *CXCL7* gene and were previously described.(21) They express ~3-fold higher hPBP levels relative to human controls. The transgenic hPF4⁺ and hPBP⁺ mice were then bred onto the respective knockout mouse background so that all studies were done using these human transgene-expressing mice in comparison to knockout littermate control mice.

The genomic type of all animals was determined by PCR as described ((21, 22) and Supplement Table 1 for *Pbp*^{-/-} and hPBP⁺ mice). All of the animals were housed at the Children's Hospital of Philadelphia (CHOP) animal facility. All procedures were performed after approval by CHOP's animal care and use committee.

Evaluation of in vivo PF4 release by megakaryocytes in bone marrow

To examine in vivo PF4 release by megakaryocytes, femurs were examined in eight-week old female *Pf4*^{-/-}, hPF4⁺, wildtype (WT), iDTR⁺ and iDTR⁻ mice. In some animals, thrombocytopenia was induced by injection of 70 µg/kg of anti-CD41 antibody (clone MWreg30, rat IgG1, κ) 24 hours prior to irradiation according to published protocols(25). In iDTR⁺ mice, megakaryocytopenia and thrombocytopenia were induced by injection of 10 ng/kg of DT (Sigma) subcutaneously for 7 days. Irradiated mice received 660 cGy using an X-RAD 320 biological irradiator (Precision Xray) through a fixed beam at 320kV 48 hours prior to being sacrificed. Femurs were removed from anesthetized mice and were fixed in formalin, decalcified, imbedded in paraffin, and sectioned and stained for mouse (m) PF4 using a rabbit anti-mPF4 antibody (R&D, clone 140910) at a 1:200 dilution or for hPF4 using a polyclonal rabbit anti-hPF4 antibody (Peprotech clone #500-P05) at a 1:2000 dilution according to previously reported protocols(26). A Rodent blocking step (Biocare Medical) was used for 30 minutes according to manufacturer's instructions and for the mPF4 antibody, a peptide-blocking step (DAKO X0909) was employed. Antigen retrieval was performed with E1 (Leica Microsystems) retrieval solution. Stained slides were

digitally scanned at 20× magnification (Aperio OS, Leica Bioimaging). Three sections were prepared from each animal, separated by 10 microns and two animals were evaluated per condition. Color thresholding was used to quantitate staining using ImageJ (NIH) by setting thresholds within a defined area outside of megakaryocytes (20 non-overlapping fields/slide field) and by defining megakaryocytes (20/slide field) for 3 areas per slide. The negative was defined by *Pf4*^{-/-} slide staining. For comparison, slides were stained for von Willebrand factor (vWF) using rabbit polyclonal anti-mvWF antibody (DAKO) at a 1:750 dilution by the histopathology core at CHOP.

Culture of bone marrow (BM)-derived megakaryocytes

Human CD34⁺ BM cells were purchased from the University of Pennsylvania Stem Cell Center core and were derived from healthy donors. BM from the tibias and femurs of 6–12 week-old male mice were isolated and used for in vitro cell culture(27). Briefly, Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) without modification was used to flush the marrow cavity of bones harvested from sacrificed mice, and the cells were then passed through a 100-µm nylon filter (BD Biosciences). For both murine and human cells, after centrifugation at 1000g, the cell pellet was resuspended in AIM-V media (Invitrogen) at a concentration of 1×10^6 cells/ml with 50 ng/ml recombinant mouse or human thrombopoietin (TPO), respectively (R&D Systems). Human megakaryocytes were exposed to mPF4 (25 µg/ml) at 11–14 days of culture and murine megakaryocytes were exposed to hPF4 (50 µg/ml) after 3–4 days of culture. In some cultures, blocking antibodies (polyclonal rabbit anti-hPF4 (Sigma-Genosys), polyclonal rabbit anti-mPF4 (Sigma-Genosys), monoclonal mouse anti-hLRP1 (Clone MA-5A6, Molecular Innovations) or receptor-associated protein (RAP, Molecular Innovations) were concurrently added to the media. After 24 hours of PF4 exposure, cells were washed with 1000 units/ml of sodium heparin (Sigma) and resuspended at 1×10^6 cells per ml in phosphate buffered saline (PBS) (Invitrogen). For release studies, megakaryocytes were washed with PBS and replated at 1×10^6 cells/ml in AIM-V and then activated by incubating with 0.05 units/ml human thrombin (Sigma) or 5 ng/ml convulxin (Santa Cruz Biotech) for 10 minutes at 37°C. To prepare cell lysates for enzyme-linked immunosorbent assay (ELISA) of PF4 levels, cells were pelleted after heparin wash by centrifugation at 1000g for 5 minutes, resuspended in PBS at a concentration of 1×10^6 cells/ml and then subjected to 3 cycles of freeze thaw to completely lyse the cells. The cellular debris was then pelleted prior to analysis of the supernatant for PF4 levels. Measurement of PF4 levels was by commercially available PF4 ELISA kits for hPF4 (Asserachrom PF4, Stago Diagnostica) and mPF4 (R&D Systems) according to manufacturer instructions.

Recombinant PF4 and PBP preparation

Recombinant mPF4 or hPBP was prepared as described(28). The mPF4 and hPBP were isolated from BL21DE30 pLysS bacterial cells. These proteins were further purified by fast protein liquid chromatography using a Resource RPC FPLC column (Amersham Pharmacia Biotech). Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. An immunoblot after electrotransfer to polyvinylidenedifluoride was used to confirm the identity of the proteins.

Human PF4 was generated as previously described from S2 insect cells(29). Briefly, cDNA encoding hPF4 was cloned into the plasmid pMT/BiP/V5-His A (Invitrogen) for expression in *Drosophila* Expression System (Invitrogen). Expression of hPF4 was induced by adding copper sulfate to 0.5 mM. Induced cells were incubated in Insect-Xpress (Lonza Walkersville) media for 3–5 more days; supernatant collected, sodium azide (0.02% final concentration) and ethylenediaminetetraacetic acid (EDTA, 2.5 mM final concentration) added, and the media filtered through an Express Plus 0.22- μ m filter (Millipore). The hPF4 was purified from the media on a heparin HiTrap column on an ATKA Prime (GE Healthcare) at 4°C in 10 mM Tris, 1 mM EDTA, pH 8.0 buffer as described(29).

LRP1 shRNA studies

Three shRNAs against mouse LRP1 were created in a lentivirus that conferred puromycin resistance (EZBiolab). Plasmid DNA for vectors was produced in DH5 α bacteria, and using a 3-vector lentivirus system, viral particles were generated in 293T cells as described(30). Virus was titered by the QuickTiter Lentivirus Quantitation Kit (Cell Biolabs), yielding titers between $0.9\text{--}1.8 \times 10^{11}$ viral particles/ml. Using primary BM obtained from *Pf4*^{-/-} mice, cells were pelleted and resuspended at a concentration of 2×10^6 cells/ml in unmodified IMDM. Approximately 500 μ l of the cell suspension was placed in a 24-well plate (Corning), and plates were spun at 340g for 5 minutes to form a monolayer of cells. Then, 4 μ l of polybrene (2 mg/ml, Millipore) was added to cells with 50 μ l of concentrated virus. Cells were spun again at 340g for 90 minutes at 20°C and then placed at 37°C for 3–4 hours. Afterwards, cells were resuspended in fresh serum-free AIM-V media with 50-ng/ml recombinant TPO. One μ g/ml puromycin (Sigma) was added after 48 hours. After 3 days, hPF4 (25 μ g/ml) was added to the culture media for 24 hours, and cell lysates were prepared and analyzed as described under *culture of megakaryocytes*.

Immunofluorescence and confocal microscopy

Immunofluorescence microscopy (IFM) followed a described protocol(31). Briefly, BM-derived megakaryocytes were collected from the culture and loaded on a bovine serum albumin (BSA) gradient (1.5%/3%) to purify mature megakaryocytes. These cells were fixed with 2% formaldehyde, prepared by cytospin and labeled with primary and fluorochrome-conjugated secondary antibodies in PBS containing 0.2% saponin and 0.1% BSA. Primary antibodies were: rabbit polyclonal anti-mPF4 antibody (Sigma-Genosys), monoclonal anti-hPF4 RTO (Abcam), mouse polyclonal anti-human PBP antibody and goat polyclonal anti-P-selectin (both, Santa Cruz Biotechnology), and rabbit polyclonal anti-mvWF (DAKO). Detection was performed with AlexaFluor 594-donkey anti-rabbit IgG, AlexaFluor 488-donkey anti-goat IgG, or AlexaFluor 488-donkey anti-mIgG (all, Jackson ImmunoResearch Laboratories). The nuclei were stained with Hoechst 33342 (0.1 μ g/ml, Invitrogen). For standard immunofluorescence microscopy and deconvolution, cells were analyzed on a Leica DM IRBE microscope (Leica Microsystems) equipped with a Retiga-SRV digital camera (QImaging) and OpenLab image acquisition software (Perkin-Elmer). Images were acquired from sequential z-stacks in 0.2 μ m steps, analyzed, processed by volume deconvolution and visualized with XY plane mode using Volocity software (Perkin-Elmer), and further processed using Adobe Photoshop (Adobe Systems). For confocal microscopy images were acquired on a Zeiss LSM 510 NLO/META confocal microscope

(Carl Zeiss Microscopy) with LSM 510 v. 4.5 software and analyzed with Volocity software. Because antibodies fail to penetrate deep into the large multinucleated megakaryocytes, only planes of cells within 5–10 μm of the coverslip were imaged. Colocalization was quantified from single planes of deconvolved images, and is presented as a thresholded Pearson's correlation coefficient from at least 5 cell profiles for each pairwise comparison. Thresholds for each channel in each image were determined by choosing several areas of blank space in the image using the "voxel pick tool" and calculating the average intensity of the signals. Saturated signals were also excluded from the analysis using the same procedure.

Statistical analysis

Statistical analysis of the non-imaging data was performed using GraphPad Prism 6 software. Data were analyzed using one-way ANOVA or Student's t-test with Bonferroni Correction (for multiple comparisons), and a p value <0.05 was considered significant. Imaging data were analyzed using Graphpad Prism 5 utilizing one-way ANOVA and the same p value cutoff for statistical significance.

Results

PF4 is released in vitro and in vivo by megakaryocytes

PF4 content influences megakaryopoiesis in mice(20, 32) and patients(33) and HSC replication.(15) These findings imply that there is steady-state free PF4 within the BM intramedullary space, and the level of free PF4 increases under stress. In support of this concept, PF4 levels in the medium during initial culture of hCD34⁺ hematopoietic cells are undetectable, but increase as the fraction of CD41⁺ megakaryocytes increases (Supplement Figure 2A) and peak as the number of megakaryocytes in the culture peaks (Supplement Figures 2A and B).

We then addressed whether PF4 release from megakaryocytes occurs in vivo. In mice, as in humans, the BM is the primary source of megakaryopoiesis. Immunohistochemistry of mouse BM using a polyclonal antibody to hPF4 in hPF4⁺ mice showed intense staining of large cells (morphological megakaryocytes), only in the hPF4⁺ mice (Supplement Figure 3, non-irradiated), not in *Pf4*^{-/-} negative controls. General background marrow staining was notable in the hPF4⁺ mice, but in addition, there was a halo of PF4 staining peri-megakaryocytes as well as intense staining of the megakaryocytes (Figure 1B and C). This contrasted with vWF-staining that was markedly limited to only within megakaryocytes (Figure 1C). Post-irradiation, the hPF4⁺ BM showed a marked decrease in PF4 staining of the megakaryocytes and an increase in the background staining (Supplement Figure 3, irradiated). Additionally, intramedullary PF4 did not come from circulating platelets, as immune-induced thrombocytopenia did not alter these findings (Figure 1A and 1D). Further, DT induction of megakaryocytopenia/thrombocytopenia in iDTR⁺ mice resulted in decreased background staining in the BM compared to iDTR⁻ mice both pre- and post-irradiation (Figure 1B and 1D), consistent with Figure 1A and with prior studies showing that ~95% of all PF4 in the BM comes from megakaryocytes(15).

PF4 is internalized by megakaryocytes

Thus the data in Figure 1 and Supplementary Figures 2 and 3 support that intramedullary PF4 is released by megakaryocytes and that additional PF4 is released after injury. What happens to the released PF4? One possibility is that a portion is re-internalized by the megakaryocytes, since these cells are known to take up and store other proteins from its surrounding(7, 9). To examine by this, we cultured BM isolated from *Pf4*^{-/-} mice and added hPF4 (50 µg/ml) for 24 hours. After washing with heparin to remove surface-bound PF4 prior to cell lysis, hPF4 was detected in cell lysates of *Pf4*^{-/-} megakaryocytes (Figure 2A). Analogous results were found when similar studies were performed using human megakaryocytes exposed to mPF4 (25 µg/ml) (Figure 2B). PF4 uptake was significantly decreased by adding blocking antibodies to PF4 or to LRP1 or by adding RAP, an inhibitor of ligand uptake by LRP1 family members(34) (Figures 2A and 2B). Stimulation of *Pf4*^{-/-} murine megakaryocytes that had been pre-incubated with hPF4 with thrombin or convulxin released the hPF4 back into the culture medium (Figure 3A) indicating that internalized PF4 is targeted to a cellular pool that can be re-released.

To address the question of how the amount of PF4 internalized compared to endogenous PF4 levels, we examined PF4 levels in WT murine megakaryocytes cultured in the presence of hPF4 and analyzed the relative concentrations of hPF4 and mPF4. The concentrations of the two proteins were similar (19 vs. 29 ng/10⁶ megakaryocytes, respectively (Figure 3B)), indicating that a significant amount of PF4 is internalized by megakaryocytes.

Internalized PF4 localizes to alpha granules

If internalized PF4 is available for release upon agonist activation (Figure 3A), we reasoned that it might be stored within alpha granules. Using murine BM-derived *Pf4*^{-/-} megakaryocytes exposed to hPF4 for 24 hours, we co-stained for antibodies to hPF4, the alpha-granule marker P-selectin,(35, 36) and the lysosome marker LAMP-1(37). Confocal microscopy showed that hPF4 partially co-localized with P-selectin (Figures 4A–C), but not LAMP-1 (Figures 4D–F), indicating that exogenous hPF4 is stored in alpha granules, but not in lysosomes. The degree of co-localization of internalized hPF4 with P-selectin was at least as extensive as that observed between P-selectin and other alpha-granule proteins such as vWF and fibrinogen (Supplemental Figure 4), but none showed full co-localization, consistent with previous reports(38)–(39).

To test whether internalized PF4 and endogenously expressed PF4 are stored in similar or separate alpha-granules, we exposed WT murine megakaryocytes to hPF4 for 24 hours, and then fixed and stained the cells with species-specific antibodies (Supplement Figure 5) before confocal microscopy analysis (Figures 5A–F). Internalized hPF4 and endogenous mPF4 partially co-localized, indicating that internalized PF4 can be trafficked to the same alpha granules in megakaryocytes as endogenous PF4. This is consistent with the known presence of both internalized (eg, fibrinogen) and expressed (eg, vWF) proteins within alpha granules.

PBP is not internalized by megakaryocytes

To begin to test whether other endogenously synthesized alpha-granule contents can also be internalized by megakaryocytes and stored in alpha-granules, we examined whether PBP undergoes similar processing. PBP is another platelet-specific chemokine and has sequence homology with PF4(40). BM isolated from *Pbp*^{-/-} mice was incubated with recombinant hPBP (as done in Figure 3 for *Pf4*^{-/-} mice and hPF4). Cells were harvested 24 hours later, washed with heparin, and processed for ELISA studies and immunohistochemistry. No hPBP was detected in *Pbp*^{-/-} megakaryocytes, after exposure to hPBP in culture, but was readily detected in megakaryocytes derived from hPBP⁺ transgenic mice (Figure 6), indicating that hPBP, unlike hPF4, is not internalized by murine megakaryocytes in vitro, and suggesting that chemokine internalization might be unique to PF4.

Requirement for LRP1 in PF4 reuptake

We have previously shown that LRP1 is required for the negative paracrine effect of PF4 on megakaryopoiesis.(20) We now assessed whether LRP1 is also involved in PF4 internalization. PF4 uptake into *Pf4*^{-/-} megakaryocytes was significantly decreased by treatment with either anti-LRP1 antibody or by RAP (Figures 2A and B), suggesting that LRP1 is at least partially required for optimal PF4 internalization. To further support these studies, we decreased LRP1 expression in megakaryocytes using an shRNA approach.(20) Consistent with our prior findings, treatment with LRP1-specific shRNA significantly decreased surface LRP1 by ~70% ($p < 0.0004$, Figure 7A). Similar to the blocking antibodies, treatment with the LRP-specific shRNA significantly decreased PF4 uptake by megakaryocytes ($p < 0.02$, Figure 7B). Interestingly, when *Pf4*^{-/-} megakaryocytes were treated with exogenous hPF4 we noted that surface LRP1 levels increased >4-fold on megakaryocytes over baseline (Figure 7A). A time-course study showed that surface expression of LRP1 increased in a time-dependent fashion in *Pf4*^{-/-} megakaryocytes exposed to hPF4, peaking at 24 hours of exposure (Figure 7C). These data indicate that LRP1 at least partially mediates PF4 uptake in megakaryocytes, and that LRP1 surface expression is increased by PF4 exposure.

Discussion

Intramedullary PF4 has clinically relevant roles in controlling steady-state platelet numbers(17) and HSC replication.(15) We now show that intramedullary PF4 is derived from megakaryocytes, not platelets, and that megakaryocytes can regulate the level of intramedullary PF4 by internalizing free PF4. Our prior studies suggest that intramedullary PF4 can influence the duration and severity of chemotherapy- and radiation-induced thrombocytopenia.(18, 41) This same pathway could potentially impact other states that are associated with chronic intramedullary injury, such as immune thrombocytopenia and myelodysplastic syndrome, where recalcitrant thrombocytopenia may be due in part to intramedullary PF4 released from injured megakaryocytes. In addition, our previous work has shown that blocking PF4 (in animal models) can significantly impact platelet count recovery(19, 41) and even survival(18) after chemotherapy and radiation injury, further demonstrating relevance of this pathway.

The presented data also suggest a complex intramedullary life cycle for PF4. In fact, our data support the possibility that PF4 may undergo more than one round of release and reuptake since the level of PF4 that can be taken up from the media by a megakaryocyte approaches its level of endogenous PF4 (Figure 3). In Figure 5, we discussed the endogenous mPF4 as if it had not undergone re-internalization in contrast to the exogenous hPF4, which clearly must undergo uptake to be within the murine megakaryocytes; however, both may have undergone uptake by the megakaryocyte, explaining their localization to common granules. Clearly additional studies are needed to quantify the kinetics of PF4 uptake and to determine whether PF4 is not all first released and then taken up into alpha granules. Such analyses may provide additional insights into alpha-granule heterogeneity(39), organization and kinetics. It is also not clear why PF4 has this complex life cycle. This points to the biological importance of intramedullary PF4 as a regulator of HSCs and megakaryopoiesis. It is also not known whether PF4 or LRP levels vary with age and whether variations in relative concentrations of these proteins may have a role in the relative frequencies of bone marrow failure and MDS (both are more common in older populations) as PF4 has been reported to have a role in myelofibrosis development in these disorders.(42)

Do other alpha-granule proteins demonstrate a similar lifecycle? PBP is structurally very similar to PF4 with ~60% sequence identity.(21) An N-terminally cleaved product of PBP, neutrophil-activating peptide 2, can also inhibit megakaryopoiesis *in vitro*.(43) However, our data show that PBP does not undergo internalization in megakaryocytes under similar conditions in which PF4 is internalized. Indeed, platelet count recovery after chemotherapy-induced thrombocytopenia is similar in *Pbp*^{-/-} and hPBP⁺ mice, which express vastly different levels of PBP (data not shown), suggesting that PBP does not impact megakaryopoiesis *in vivo*. Additionally, histological studies in Figure 1c suggest that vWF is not released intramedullary in the steady-state. If release and reuptake is unique to PF4, general alpha-granule release by stressed megakaryocytes would result in a significant loss of other granular content both in megakaryocytes and in derived platelets, while PF4 is taken back up. The end result might be low alpha-granular content in platelets from stressed megakaryocytes, especially relative to PF4 content, and an accumulation of other granular proteins in the intramedullary space. Recently *Nbeal2*^{-/-} megakaryocytes were shown to lose their alpha-granule content(44). We predict that these megakaryocytes lose the ability to recycle PF4, but whether the released PF4 contributes to the observed thrombocytopenia is unknown.

PF4 internalization is at least partially dependent on LRP1 on the megakaryocyte surface. PF4 uptake was reduced by using anti-LRP1 antibody or RAP and by decreasing surface LRP1 levels by shRNA treatment. Similar effects of LRP1 reduction or inhibition on uptake were previously observed for factor V in human megakaryocytes.(8) LRP1 is responsible for the internalization of numerous ligands, including factor VIII, on endothelial and dendritic cells(45, 46). In these cases, internalization leads to lysosomal degradation of the ligand and recycling of the LRP1 to the cell surface following ligand-receptor uncoupling in early endosomes.(8, 46) However, our data suggest that PF4-bound LRP1 follows a distinct

pathway following internalization such that LRP1 is recycled and PF4 is not targeted for degradation, analogous to the fates of EGF receptor binding to distinct ligands.(47)

In summary, intramedullary PF4 regulates HSC and megakaryopoiesis. We now show that this PF4 comes from megakaryocytes during steady-state and that this level increases following marrow injury. Additionally, released PF4 can be significantly internalized into megakaryocyte alpha-granules, in part, via LRP1, perhaps as part of a physiologic feedback loop regulating megakaryocyte and HSC number, but this needs further study. Moreover, whether this intramedullary recycling process is unique to PF4 or is seen with other megakaryocyte-derived proteins warrants investigation. At the moment, *Nbeal2*^{-/-} megakaryocytes appear to release alpha-granular proteins, but whether other endogenous alpha-granule proteins are normally released and re-internalized, and how this pathway affects alpha-granule heterogeneity in various clinical settings needs to be examined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunohistochemistry of thrombocytopenic and megakaryocytopenic murine bone marrow

Representative sections of bone marrow are shown immunostained for (A) hPF4 or (B) mPF4, where on the left are non-irradiated mice studies and on the right, irradiated. (A) Immuno-thrombocytopenia was induced to a level of $207 \pm 95 \times 10^6/\text{ml}$ vs. $1252 \pm 87 \times 10^6/\text{ml}$ in the untreated ($p < 0.001$) in *Pf4*^{-/-} mice and to $286 \pm 104 \times 10^6/\text{ml}$ vs. $1087 \pm 99 \times 10^6/\text{ml}$ in the untreated ($p < 0.001$) in *hPF4*⁺ mice by injection of antiCD41 antibody (25) daily for 5 days prior to irradiation, after which bone marrow was harvested and studied for PF4 distribution. (B) Megakaryocytopenia/thrombocytopenia was induced in *iDTR*⁺ and *iDTR*⁻ mice after DT injection. Platelet counts after DT injections in *iDTR*⁺ mice were significantly lower ($163 \pm 13 \times 10^6/\text{ml}$) than prior ($684 \pm 37 \times 10^6/\text{ml}$, $p = < 0.001$). In *iDTR*⁻ mice, DT injection had no effect ($964 \pm 22 \times 10^6/\text{ml}$ post-DT vs. $1004 \pm 90 \times 10^6/\text{ml}$ pre-DT, $p = 0.693$). In (A) and (B), arrows point to representative megakaryocytes. Bars = 100 μm . (C) Staining of WT bone marrow with anti-vWF antibody (top panel) or anti-mPF4 antibody (bottom two panel) showing in addition to decreased background staining for PF4 only, sharply demarcated staining of megakaryocytes in vWF stained slides and a halo of stained megakaryocytes in PF4 stained slides. Inset shows highlighted megakaryocytes at increased magnification (100 $\times$) demonstrating the distinction of peri-megakaryocyte staining. (D) Relative quantitation of staining of megakaryocytes (top) and area between megakaryocytes (bottom) using the color thresholding technique in ImageJ. Mean $\pm$ SD shown. Significant p values are highlighted and were calculated after correction for multiple comparisons. Of particular note, in DT-treated *iDTR*⁺ mice in contrast to the DT-treated *iDTR*⁻ mice, megakaryocytes were few (B) and contained little PF4, and this level did not decrease after irradiation (D, top). Inter-megakaryocyte free PF4 was low in the non-irradiated *iDTR*⁺ mice, and this level did not increase after irradiation in contrast to the increase in *iDTR*⁻ mice (D, bottom).

Figure 2. PF4 uptake in murine and human megakaryocytes

(A) hPF4 uptake by *Pf4*^{-/-} megakaryocytes. *Pf4*^{-/-} BM-derived megakaryocytes after 3 days in culture were untreated (left lane) or exposed to hPF4 (50 µg/ml in all other lanes) alone or with concurrent RAP (2 ng/ml), or with blocking antibody to PF4 (α-PF4) or to LRP1 (α-LRP1; 25 µg/ml) for 24 hours. Values represent mean ± standard error of the mean (SEM); number of experiments (n) and p values are shown. (B) As in (A) but for human bone marrow CD34⁺ cell-derived megakaryocytes (hMegs) cultured for 7 days after which no PF4 (left lane) or mPF4 (25 µg/ml in all other lanes) was added with or without α-PF4 antibody or RAP.

Figure 3. Measurement of PF4 release and mouse and human PF4 in WT megakaryocytes at 24 hours

(A) Murine *Pf4*^{-/-} cells were incubated with thrombin (black and white bars) or convulxin (gray bars). Black and dark gray bars indicate PF4 levels measured by ELISA in the culture supernatants. White and light gray bars present PF4 levels measured in cell lysates. Mean $\pm$ SEM shown. N = 3 separate experiments, each in duplicate. (B) WT murine megakaryocytes were cultured in the presence of hPF4 (50 μ g/ml) and after 24 hours were harvested, washed with heparin and PBS and then subjected to 3 freeze/thaw cycles to lyse the cells. The cell lysates (1×10^6 cells/ml) were then analyzed by ELISA for PF4 concentrations of both mPF4 and hPF4.

Figure 4. PF4 localizes to alpha granules and not to lysosomes in *Pf4*^{-/-} megakaryocytes
Pf4^{-/-} BM-derived megakaryocytes were exposed to hPF4 (A–F) or not (G–I), and then fixed, permeabilized and processed for immunofluorescence confocal microscopy. A–C and G–I, cells were labeled for mP-selectin (green) and hPF4 (red); merged images in C and I. D–F, cells were labeled for mLAMP-1 (green) and hPF4 (red); merged image in F. Insets show 3.5 × (A–C) or 2.75 × (D–F) magnification of boxed regions. Bars = 10 μm.

Figure 5. Colocalization of PF4 internalized by large and small WT murine megakaryocytes and endogenous PF4

BM-derived megakaryocyte cultures from WT-mouse were exposed to hPF4 and then fixed, permeabilized and processed for immunofluorescence confocal microscopy using antibodies specific to hPF4 (green, A and D) and mPF4 (red, B and E). Merged images are shown in C and F. Insets show 3 × magnification of boxed regions. Bars = 10 μm.

Figure 6. PBP uptake in murine megakaryocytes

(A–F). BM-derived megakaryocyte cultures from *Pbp*^{-/-} mice exposed to hPBP (A–C) or from hPBP⁺ transgenic mice (D–F) were fixed, permeabilized and labeled with antibodies to mvWF (red; A, D) and hPBP (green; B, E) and DAPI to label nuclei (blue). Merged images are shown in (C) and (F). (G) BM-derived megakaryocytes from hPBP⁺ transgenic (first bar) or *Pbp*^{-/-} mice were exposed to hPBP (50 mg/ml) for 24 hour. Cell lysates were then analyzed by ELISA assay for hPBP. Mean ± 1 SEM shown. N = 3 experiments, each done in duplicate.

Figure 7. Effect of anti-LRP1 shRNA on LRP1 expression and PF4 uptake

(A) Murine WT megakaryocytes treated with control shRNA or LRP1-specific shRNA, and exposed to hPF4 (50 mg/ml). Mean fluorescence intensity (MFI) $\pm$ 1 SEM for surface LRP1 is shown. Dashed line represents the MFI of isotype control. (B) Lysates of cells treated with control or LRP1-specific shRNAs and exposed to hPF4 were analyzed by ELISA to assess hPF4 uptake. (C) WT megakaryocytes were treated with hPF4 for the indicated times, and then labeled with FITC-anti-LRP1 and analyzed by flow cytometry. Shown is the MFI; dashed line represents MFI of the isotype control. For (A) through (C), mean $\pm$ SEM is shown. N = 3 experiments per arm, each done in duplicate.