Recombinant human PF4 (rPF4): rPF4 was expressed in BL21(DE3) *E.coli* using cDNA expressed in the PET-24a(+) vector (Novagen). The original cDNA was kindly provided by Dr. Mortimer Poncz (University of Pennsylvania). Point mutations in PF4 (PTA37-39AVP, R49S, L55R, L11V and E4S) were introduced using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). Recombinant wild-type (WT) and mutant products were purified as described elsewhere[1-3]. The avidity of each mutant and wild-type PF4 for heparin was evaluated using FPLC (GE-Healthcare, Piscathaway, NJ) by comparing the elution profile with platelet-derived PF4. In a NaCl gradient from 0.145 to 2.2M in PB (phosphate buffer), all WT and rPF4 eluted between 1.4-1.6 M NaCl. ELISA experiments performed using rabbit polyclonal anti-hPF4 confirmed that the FPLC peaks correspond to PF4. Further, these products migrated as a single ~8kDa band in SDS-PAGE under reducing conditions. The tetrameric status of WT and mutants was verified using HPLC size exclusion chromatography under isocratic conditions (20mM PBS-NaCl 0.145M) using Spherogel TSK 4000SW column (Beckman-Coulter). Ovalbumin (43kDa) and cytochrome-C (12.4kDa) were used as size standards, along with platelet derived hPF4 in native form, and monomer hPF4 formed by desalting tetramer and resuspension of precipitate in 0.5% (v/v) pyridine, 10% acetic acid, pH 3.15.

Rat PF4: Blood from 25 male Sprague Dawley (CD) rats drawn into acid-citrate-dextrose (ACD) following cardiac puncture was used as a source of rat PF4. The purification strategy for rat PF4 was similar to that for human PF4, with minor modifications. Briefly, PRP (platelet-rich plasma) was obtained within 3h of blood collection by centrifugation at 200g for 15min at room temperature (RT). Platelets were pelleted by further centrifugation of PRP at 2500g for 15min.

The pellet was washed thrice in Ringer's citrate dextrose buffer containing 2mM EDTA, and resuspended in 2ml PBS containing 1.4mM PMSF. This platelet suspension was subjected to three freeze-thaw cycles, and supernatant was recovered after further centrifugation at 14,000g for 20min. Ammonium sulfate was added to this supernatant at 50% saturation. Following overnight incubation at 4˚C, any precipitate formed was discarded and the final supernatant was exhaustively dialyzed against PBS. This dialyzed supernatant was incubated with 2ml heparin-agarose beads (GE Healthcare) for 4h at 4˚C, and then washed with three volumes of PB-NaCl 0.145M, PB-NaCl 0.8M (to remove "low avidity" heparin binding proteins) and 1ml PB-NaCl 1.6M three times to release bound PF4. The final eluate was concentrated using 10kDa cutoff centricon filters, dialyzed against PBS at 4˚C and treated for 20 min with excess ecteola cellulose equilibrated in PBS to remove residual contaminants. ELISA and SDS-PAGE were performed as described above to confirm purity of the eluted PF4.

All PF4 preparations were endotoxin-free as determined using the Limulus Amebocyte Lysate (LAL) assay (Cambrex, Walkersville, MD). Protein concentrations were determined using micro-BCA assay (Pierce). PF4 concentrations are expressed in μM units (7.8μg/ml=1μM monomeric PF4).

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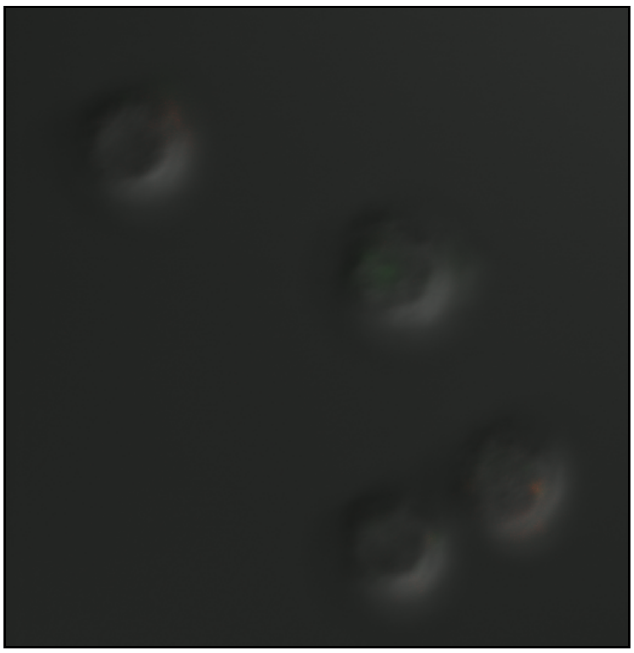
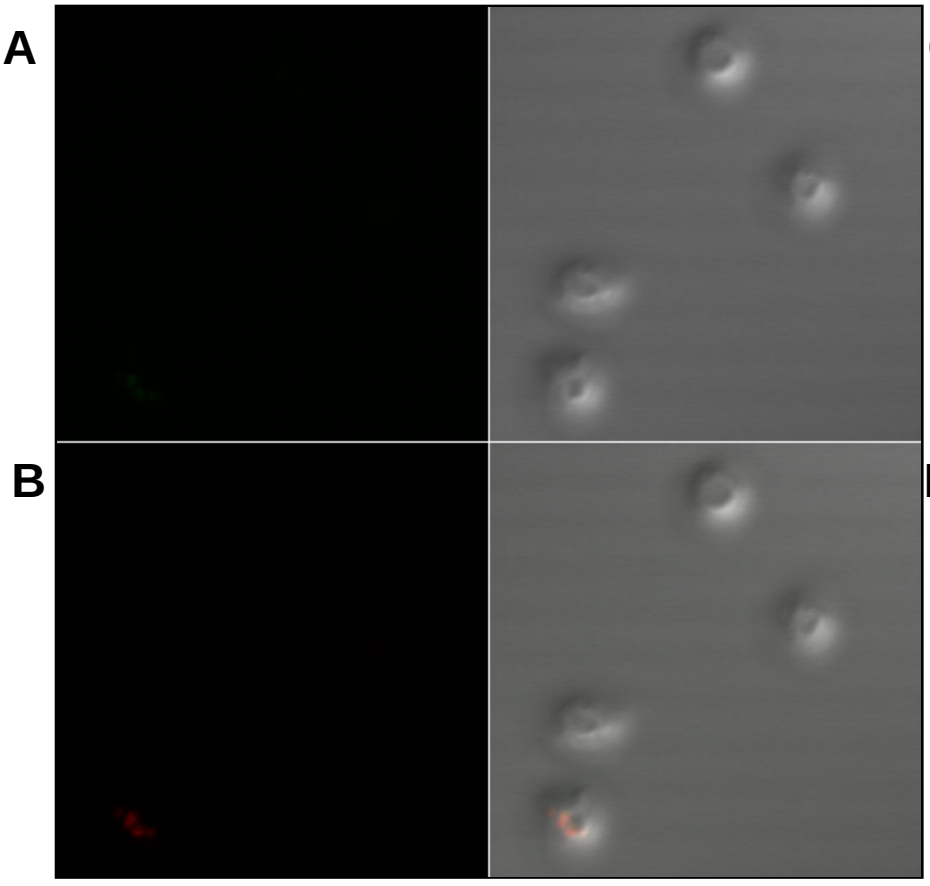
3. Liu CY, Battaglia M, Lee SH, Sun QH, Aster RH, Visentin GP. Platelet factor 4 differentially modulates CD4+CD25+ (regulatory) versus CD4+CD25- (nonregulatory) T cells. J Immunol. 2005;174:2680-2686.

Two representative confocal movie-stacks demonstrating colocalization of anti-PF4 antibody 197.3 and CD32a antigen on isolated human neutrophils (please click on images to play embedded movies)



Movie Stack 1: Anti-PF4 appears in green, CD32a appears in red, and colocalization signal appears in yellow.



Movie Stack 2: A. Anti-PF4 channel; B. Anti-CD32 channel; C. DIC image; D. Merged data from panels A-C.