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Autoantibodies immuno-mechanically modulate platelet contractile force and bleeding risk

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Altered mechanotransduction has been proposed as a putative mechanism for disease pathophysiology, yet evidence remains scarce. Here we introduce a concept we call single cell immuno-mechanical modulation, which links immunology, integrin biology, cellular mechanics, and disease pathophysiology and symptomology. Using a micropatterned hydrogel-laden coverslip compatible with standard fluorescence microscopy, we conduct a clinical mechanobiology study, specifically focusing on immune thrombocytopenia (ITP), an autoantibody-mediated platelet disorder that currently lacks a reliable biomarker for bleeding risk. We discover that in pediatric ITP patients ($n = 53$), low single platelet contraction force alone is a "physics-based" biomarker of bleeding (92.3% sensitivity, 90% specificity). Mechanistically, autoantibodies and monoclonal antibodies drive increases and decreases of cell force by stabilizing integrins in different conformations depending on the targeted epitope. Hence, immuno-mechanical modulation demonstrates how antibodies may pathologically alter mechanotransduction to cause clinical symptoms and this phenomenon can be leveraged to control cellular mechanics for research, diagnostic, and therapeutic purposes.

Over the past several decades, the field of mechanobiology has established that cells sense and physiologically respond to their mechanical microenvironment[1–3]. The mechanical microenvironment or extracellular matrix modulates numerous important cellular behaviors such as adhesion[4–7], spreading[8,9], fate[2,10–13] and migration[14]. Accordingly, altered mechanotransduction also likely plays a key role in the pathogenesis of many diseases, including cardiovascular disease[3] and cancer[15,16]. Typically, these are caused by long term

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changes to systemic and microenvironmental factors, such as tissue stiffness[15,17] and viscosity[18] or vessel geometry induced disturbed blood flow[3]. An area of active inquiry centers around investigating whether aberrant mechanotransduction within the cell of interest itself plays a more direct key role in the pathophysiology and symptomology of disease, as hypothesized previously by others[19,20]. For instance, recent work on pericytes have implicated aberrant pericyte contraction in exacerbating the severity of Alzheimer's Disease[21] and stroke[22]. In addition, force generation is elevated in smooth muscle cells from fatally asthmatic patients[23]. However, a key barrier to progress has been the labor-intensive approaches needed for such studies, and the few technologies capable of measuring single cell mechanotranduction[23,24]. To that end, we developed a widely accessible contraction cytometer, effectively a hydrogel on a glass coverslip that can be used with any fluorescence microscope.

Using our cytometer, we investigated whether impaired cellular biophysical function, is implicated in pediatric immune thrombocytopenia purpura (ITP), an autoimmune bleeding disorder that remains problematic for clinicians[25]. ITP is characterized by platelet destruction and consumption and is clinically diagnosed as a platelet count $<100 \times 10^9$/L with other causes of thrombocytopenia excluded. Although the etiology for ITP is unknown, canonically, the pathophysiologic process is thought to be triggered by post-infectious autoantibodies after a viral or bacterial infection[26]. For the over 12,000 individuals affected by ITP each year[27], the clinical challenge lies in predicting which of the minority of patients need immediate initiation of therapy due to risk of life-threatening bleeding. While ITP patients have an increased bleeding risk, due in part to the low circulating platelet concentrations, only a subset of patients are at risk for severe bleeding, where 20% of patients are at risk for major bleeding episodes and 1 in 200 are at risk for life-threatening intracranial hemorrhage[28]. Unfortunately, no biomarker objectively and reliably correlates with bleeding to guide treatment and predict clinical outcomes. Even low platelet counts, no matter how extreme, only loosely correlate with risk and severity of bleeding[29–31], however when significant bleeding does occur, patients typically present with platelet counts $<20 \times 10^9$/L[32], where 90% of intracranial hemorrhaging events occur[33]. The lack of a biomarker is problematic as the mainstay of ITP therapies involve immunosuppression and/or splenectomy, which harbor significant side effects. Taken together, clinicians are forced to balance the potential increased bleeding risk with significant side effects of current therapies[34], which may not even be necessary since the majority of patients spontaneously resolve without intervention. As hemostasis is an inherently mechanical process where platelets must perform multiple biophysical processes to close a wound[35], we hypothesized that single platelet force may be associated with bleeding. Previously, we generated evidence suggesting that low platelet force was associated with unexplained bleeding in a small cohort of 3 patients[24].We hypothesized that a similar phenomenon may occur in ITP. Here we report on the largest single cell biophysical biomarker study to date and show that force is a sensitive and specific marker of bleeding in ITP in 53 patients. Leveraging this technology, we also performed a detailed mechanistic study into the origin of low force and mechanistic consequence of antibodies on cellular function.

Reports of autoantibodies towards or reactive with integrins have been reported in a number of disorders including autoimmune diseases such as immune thrombocytopenia[36], ulcerative collitis[37], systemic lupus erythematosus[38], psoriasis, and psoriatic arthritis[39], as well as infectious diseases including Dengue[40], HIV[41], and hepatitis C[42]. Similarly, many ITP patients possess autoantibodies that are towards or reactive with integrins[43]. However, little is actually known about the clinical relevance of these integrin antibodies in regard to disease pathophysiology. Canonically, autoimmune antibodies effector functions include neutralization, opsonization, sensitization for killing by NK cells, sensitization of mast cells, or complement activation[44]. Separately, it is also established that generated monoclonal antibodies can stabilize integrins in varying conformations with different affinities: high affinity (extended-open), intermediate affinity (extended-closed), and low affinity (bent)[45]. Such varying states are associated with differing biophysical function including supporting or precluding adhesion[46] as well as recent research showing that intermediaries play a role in platelet aggregation[47]. However, whether autoantibodies stabilize different conformations and whether these conformations influence pathophysiology remains unknown. Prior work has suggested that asymptomatic ITP patients have enhanced platelet function[48–50] or activating antibodies in case studies[51,52]. Other studies have shown that platelet aggregation is impaired by autoantibodies in ITP[53,54], or that markers of platelet activation are decreased (PAC-1 binding and P-selectin expression)[29,55]. Our work helps show how both phenomena can occur in the context of ITP. In our investigation of the effects of antibodies to integrins on cellular force, we discovered that antibodies stabilize integrins in various conformations, and that this antibody-integrin complex can modulate single platelet forces up and down on a continuum. Hence, our work shows a unique mechanical effector function of autoantibodies that modulates cellular mechanotransduction and strongly influences the pathophysiology and symptomology of a disorder.

Results

As a first step in our investigation, we simplified the assembly and operation of our previously published well characterized 2D cellular contraction cytometer[24] into a broadly usable hydrogel-based micropatterned coverslip to make it feasible for translational research (Fig. 1a) and enable any laboratory to generate force cytometry data. The surface of the hydrogel-coverslip is micropatterned with approximately one million pairs of fluorescently labeled "microdots" bio-conjugated with extracellular matrix. Platelets in suspension incubated onto our system will attach to the microdot pairs and contract the dots together, although they occasionally spread beyond the geometric boundaries of the microdots. The applied cellular contractile force is directly proportional to the displacement of the dots (Fig. 1b). To use the system, a cell suspension is simply placed atop and incubated onto the hydrogel-laden coverslip and imaged (Fig. 1c), which can be measured *en masse* with fluorescence microscopy (Fig. 1d, e, Supplementary Figs. 1–3). Compared to our original microfluidic design[24] our newly designed hydrogel laden coverslip provides streamlined assembly, improving the production speed by 16x that of the original cytometer (Fig. 1f).

Cell contraction force is a potential clinical biophysical biomarker

In our investigation of 53 pediatric ITP patient samples (Supplementary Table 1), we discovered that low platelet contractile forces strongly correlate with bleeding severity and is therefore a potential biophysical biomarker of bleeding. Patient bleeding was quantified with the Buchanan bleeding score[56], which relies on a variety of visual markers to grade patient bleeding from 0 (none) or 1 (minor) to 4 (severe) or 5 (life-threatening). Specifically, our data show that platelets from ITP patients with higher bleeding scores consistently lack highly contractile subpopulations of platelets (Fig. 2a). When the platelet contractile force of each patient's platelets is averaged, we observed a significant difference between the average platelet contractile force in patients with bleeding scores <2 compared to patients with bleeding scores ≥2 (Supplementary Fig. 4). Furthermore, platelets from patients with bleeding scores of 1 or 0 exerted average contractile forces of ~35 nN, and in contrast, platelets from patients with higher bleeding scores of 2, 3, 4 exhibited average contractile forces of 18.8 nN, 21.6 nN, and 18.4 nN, respectively (Fig. 2b). As both platelet count and mean platelet volume have been used as possible biomarkers of bleeding[57], we find that neither platelet count nor platelet

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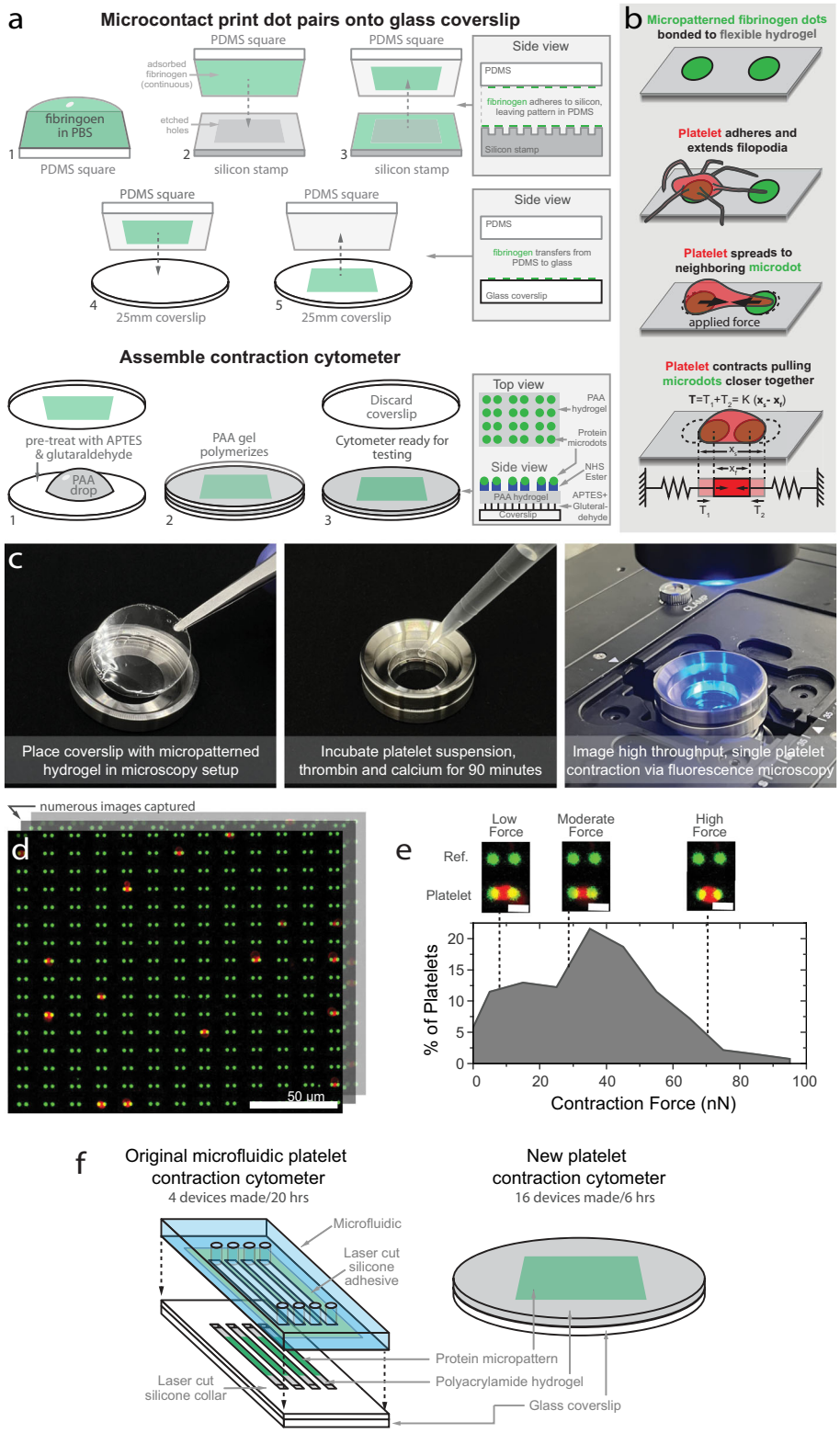

volume provide substantial stratification of patients with different Buchanan bleeding scores, however platelet counts >40 K/μL seem to greatly decrease bleeding risk (Fig. 2c, d, Supplementary Table 2). Although an extremely sensitive marker of bleeding risk, platelet count is not as specific since a substantial population of patients have extremely low platelet counts (<20 K/μL) but little to no bleeding (Fig. 2c). Additionally, with a regression tree and only leveraging platelet contraction cytometry, we found that average platelet contractile forces of <25.2 nN best predict bleeding scores ≥2 with a clinical sensitivity of 92.3% and a specificity of 90%.

Upon further analysis, we found using both platelet force and platelet count as biomarkers further improves prediction of moderate to severe bleeding scores. In our cohort, while four ITP patients with average platelet contraction forces <25.2 nN exhibited little to no bleeding, three of these patients had platelet counts >40 K/μL. To that end, when platelet count and platelet contraction force are plotted

Fig. 1 | Simplified platelet contraction cytometer enables any lab to perform high-throughput measurements of single-cell forces toward clinical translation. a Our cellular contraction cytometer has been simplified to comprise a microscope coverslip functionalized with a polyacrylamide hydrogel micropatterned with fluorescent extracellular matrix proteins. **b** Individual platelets adhere, spread, and contract against the micropatterned fibrinogen microdot pairs on the hydrogel. The microdots act akin to a spring, which enables straightforward calculations of platelet contractile force at the single cell level using measurements of the microdot size and spacing. **c** This micropatterned coverslip is adaptable for use with any fluorescence microscope. **d** The large array of fibrinogen microdot pairs enables the collection of numerous images and, therefore, allows measurements of platelet contraction forces to be captured. Scale bar is 50 μm. **e** The histogram is representative of a healthy adult, with a distribution of low, moderate, and highly contractile platelets. Scale bar denotes 4 μm. **f** Our micropatterned hydrogel-laden microscope coverslip-based cellular contraction cytometry technology allows for platelet contraction cytometers to be produced at a rate of 3.33 devices/h, a stark improvement from our original microfluidic system[24], which had a production rate of 0.2 devices/h. The microscope image in e is representative of at least 20 images collected for each of the 53 patient samples and 30 healthy donors, and the histogram in *f* is representative of 30 histograms collected for healthy donors.

together, patients can be stratified into having different bleeding risks. We show that patients with a bleeding score ≥2 generally possessed platelet contractile forces <25.2 nN and platelet counts <40 K/μL, suggesting that low values for both parameters are indicative of a "major risk" of bleeding. Patients with either low platelet contractile force or low platelet count had a "minor risk" of bleeding and consistently exhibited bleeding scores of 0 to 1 (Fig. 2e). Taking into consideration both platelet count (<40 K/μL) and platelet contractile force (< 25.2 nN), the clinical specificity improves to 97%. Combining both average platelet contraction force and platelet volume did not yield a similar stratification as a subset of patients with bleeding scores ≥2 possessed platelets with high mean platelet volume (MPV) while others possessed platelets with low MPV (Supplementary Fig. 5). Additionally, we examined the diagnostic value of platelet contraction force, platelet volume and platelet count alone in addition to combinations of platelet contraction force, platelet count and platelet volume via receiver operator characteristic (ROC) analysis (Fig. 2f), which enables an unbiased visualization of combined sensitivity/specificity as the area under the curve (AUC), to predict bleeding scores ≥2 (Fig. 2f, Supplementary Fig. 6) and ≥3 (Supplementary Fig. 7, Supplementary Table 3). Between platelet contractile force, platelet count, and MPV, platelet contractile force was a significantly stronger predictor of bleeding, with an AUC of 0.95 (with AUC = 1 being an "ideal" test) for predicting bleeding scores ≥2 (Supplementary Table 4). Platelet count and MPV were associated with much lower AUCs of 0.82 and 0.64, respectively, whereas an AUC of 0.99 was achieved by combining platelet count with platelet contractile force (Fig. 2f, Supplementary Table 4), combining platelet contraction force and platelet volume lead to no significant improvement over force alone, and an AUC of .99 was achieved by combining all 3 biomarkers (Supplementary Fig. 6, Supplementary Table 4). As such, our data collectively shows that the development of biophysical assays that allow for platelet functional measurements such as contraction could supplement platelet count and MPV to better predict bleeding risk in ITP patients. Such assays must be able to accommodate the varying platelet counts that occur in ITP, which is challenging with traditional bulk assays.

We next performed longitudinal studies in a subset of our ITP patient cohort and show that changes in platelet contractile force are associated with the appearance or resolution of bleeding symptoms and that the platelet contractile force of an individual can change over time. Specifically, in seven ITP patients from whom we were able to obtain serial measurements, increases in platelet contractile force and platelet count were associated with reduced bleeding, while decreases in platelet contractile force and platelet count were associated with increased bleeding (Fig. 3). Collectively, these data raised the possibility that an extrinsic factor that changes over time was modulating platelet contractile force in ITP.

Antibodies modulate single cell contraction force

Upon identifying that low platelet contractile force is the only major difference between ITP patients with bleeding compared to those without, we then conducted studies to investigate the underlying mechanisms. Autoantibodies have long thought to be integral to the pathophysiology of ITP, and are known to target platelet antigens[36], predominantly targeting $\alpha_{IIb}\beta_3$ (70–80%), GP1b complex (20–40%), or both[43]. Platelet destruction then occurs via antibody-mediated destruction in the spleen via macrophages in Fc-dependent manner[58] or Fc-independent desalination and platelet clearance in the liver via hepatocyte Ashwell-Morell receptors[59]. However, using autoantibodies for diagnostic purposes has been historically problematic. Autoantibodies are often undetectable in ITP patient plasma and even autoantibodies that are detectable in patient plasma exhibit little correlation to bleeding, which we confirmed by sending patient platelet poor plasma (PPP) samples with varying bleeding scores to Versiti to undergo a platelet antibody screen (Supplementary Fig. 8).

Our studies first revealed that polyclonal IgG platelet-associated (PA) antibodies isolated from 5 ITP patients (Fig. 4a) directly modulated the contractile forces of healthy donor platelets in an integrin-conformation dependent manner. Using PPP from 5 patients confirmed to possess IgG platelet reactive antibodies, we isolated their IgG antibodies and incubated them on to healthy donor platelets (Fig. 4a). Antibodies derived from the PPP of symptomatic ITP patients (Patients 3–5) with higher bleeding scores of 3 and 4 decreased contractile forces of platelets from healthy donors by 17%, 20%, and 10%, respectively. Moreover, we also observed that antibodies isolated from 2 ITP patients (Patients 1 and 2) with high platelet contractile forces and a low bleeding score of 1 directly increased platelet contractile force of healthy donor platelets by 32% and 4% (Fig. 4b, c, Supplementary Fig. 8, Supplementary Table 4). As the majority of PA antibodies in ITP are directed against the integrin $\alpha_{IIb}\beta_3$[60] and because our system uses fibrinogen, the ligand for $\alpha_{IIb}\beta_3$, we then performed negative stain electron microscopy (EM) to determine if the PA antibodies from ITP patients bind to this integrin. Anti-$\alpha_{IIb}\beta_3$ antibodies were detectable in all ITP patient samples used for these experiments and interestingly, we found that the antibodies bound to and stabilized integrins into different conformations. Patients with bleeding symptoms and lower platelet contractile forces had antibody-integrin complexes in only the bent and extended closed conformations. In contrast, the patient with high forces and a low bleeding score had a substantial population of antibody-integrin complexes in the extended-open conformation (Fig. 4d). A limitation of this approach, however, is that total patient IgG antibodies were used and not just PA IgG antibodies. It has been postulated that the concentration of specific antibodies in polyclonal sera is thought to be about 1/100 of the total IgG antibody[61]. As such, the exact concentration of the subset of PA IgG antibodies within each patient is unknown. Additionally, a subset of the ITP patients had undetectable levels of platelet reactive antibodies (Supplementary Fig. 8) in their plasma and as such experiments could not be run on these samples. As such more experiments are necessary to confirm these findings and also identify other mechanisms. However, while inhibitors and antibodies have recently been shown to stabilize integrins into specific conformations[62], our work demonstrates the clinical ramifications of antibodies binding and stabilizing integrin conformations in the pathophysiology of a disease process especially at the single cell level. Specifically, we show that PA antibodies can immuno-mechanically modulate single platelet

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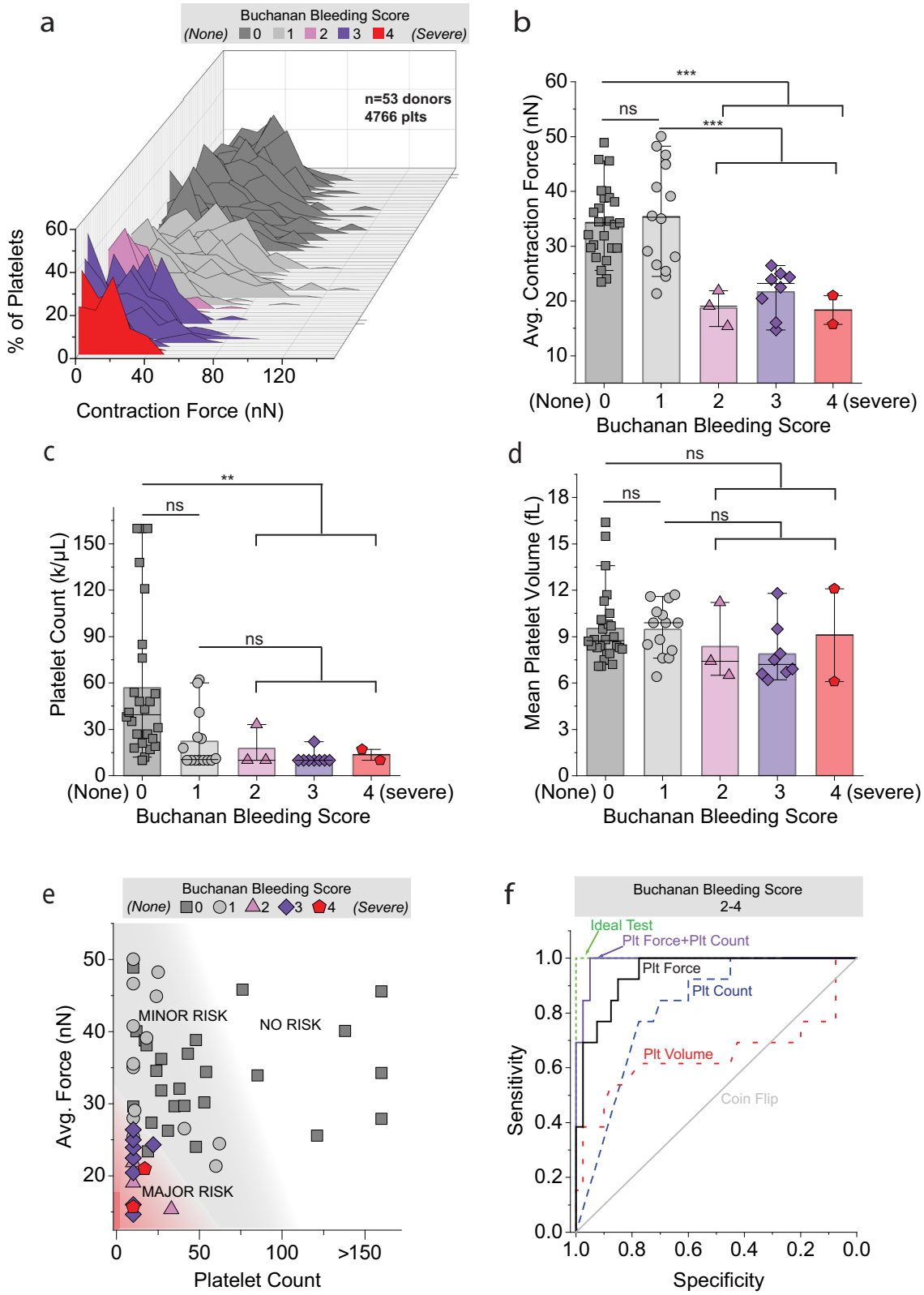

contractile force, both up and down, in an integrin conformation and epitope-dependent manner, which ultimately correlates to a patient's bleeding severity in ITP.

Based on the results of our patient polyclonal antibody experiments, we sought to better understand the relationship between conformation and epitope-dependent platelet contractile force modulation with well-characterized monoclonal antibodies targeted to different epitopes of $\alpha_{IIb}\beta_3$ (Fig. 5a). We found that it was possible to modulate platelet contractile forces both up and down, from a maximum increase of 48% (+14.4 nN) to maximum decrease of 54% (−16.3 nN). Monoclonal antibodies that bind toward the head region decrease platelet contractile force, while antibodies that bind to the tail region increase platelet contractile force (Fig. 5b, Supplementary Fig. 10, Supplementary Table 4). Negative-stain EM studies with monoclonal antibodies

Fig. 2 | Platelet contraction force is a potential clinical biophysical biomarker for bleeding risk in immune thrombocytopenia (ITP). a ITP patients ($n = 53$) with bleeding scores of 2–4 consistently lack highly contractile platelets as compared to patients with little or no bleeding. **b** Statistically, the average platelet forces of patients with moderate-to-heavy bleeding were all significantly lower than patients with no bleeding symptoms ($n = 53$) **c** Platelet count, which is currently the main clinical biomarker used in ITP, no matter how low, is not significantly associated with Buchanan bleeding scores, as a substantial population of patients have extremely low platelet counts (<20 K/μL) but little to no bleeding ($n = 53$). **d** Mean platelet volume, a surrogate marker of platelet size and immaturity, has no association with bleeding, as patients with higher bleeding scores of 2–4 possess both low and high platelet volumes ($n = 53$). As such, platelet force best stratifies low bleeding score patients from higher bleeding score patients. **e** When platelet force is considered in the context of platelet count and clinical bleeding severity score, platelet force and platelet count synergistically stratify patients by clinical severity, whereas patients with platelet counts <40 K/μL and platelet forces below 25.24 nN have an increased likelihood of having higher bleeding scores ($n = 53$). **f** Historically, platelet count and platelet volume have been proposed as biomarkers for bleeding in ITP. To compare these benchmarks to platelet force, we performed an ROC analysis, and found that platelet force alone has a high diagnostic value for predicting bleeding scores of 2–4, as demonstrated by an area under the curve (AUC) of 0.95 (CI: 0.89–1.0), exceeding both platelet count 0.82 (CI: 0.71–0.93) and platelet volume 0.64 (CI: 0.42–0.87). An improved biomarker with an AUC of 0.99 (CI:0.98–1) can be achieved by combining platelet contractile force and platelet count. Statistical significance was determined by one way ANOVA with Tukey's post hoc test for pairwise comparisons. Whiskers for 2b–d denote mean, 10th and 90th percentiles. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$ and specific p-values are shown in Supplementary Table 2. Source data are provided as a Source Data file.

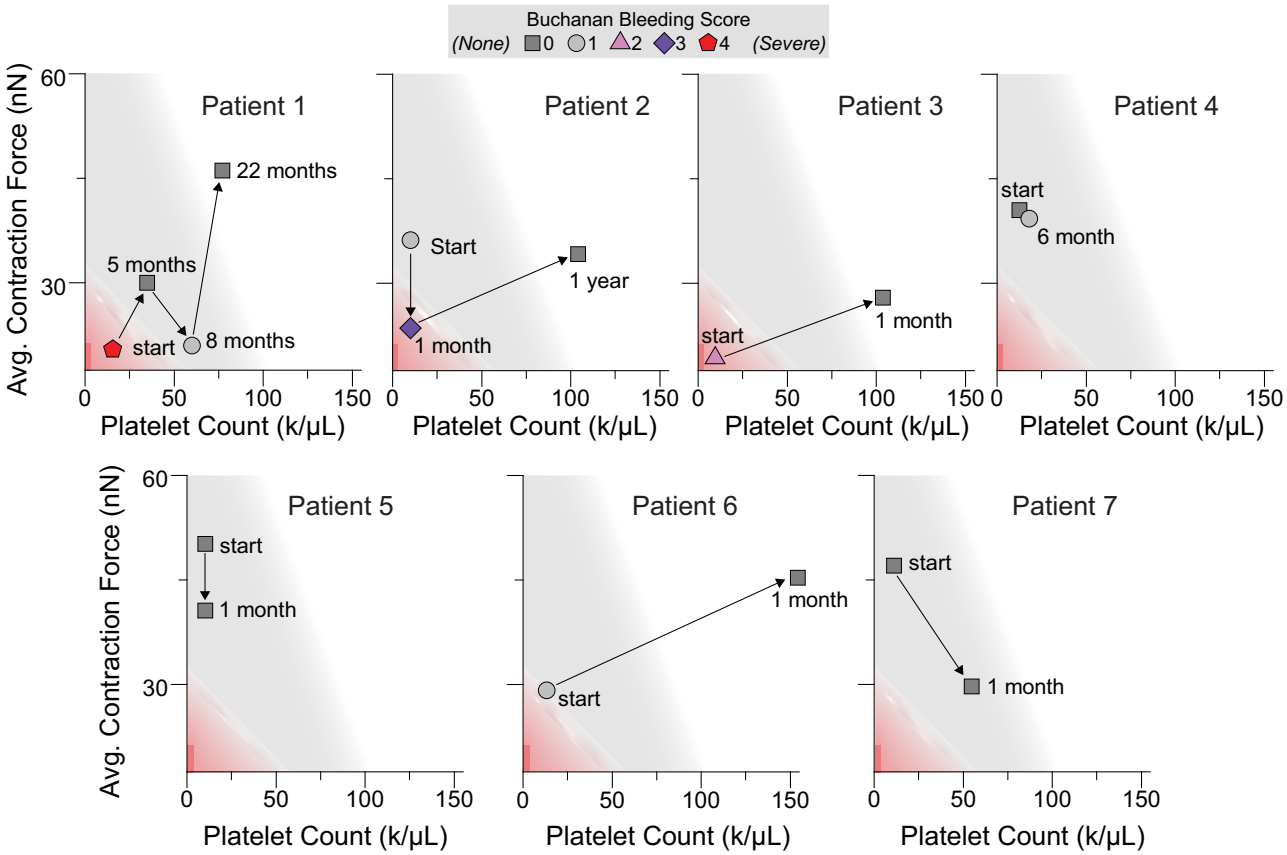

Fig. 3 | Changes in platelet contractile force are associated with the onset or resolution of bleeding symptoms in patients over time. For several patients, we examined their platelets at multiple time points and observed that platelet forces as well as platelet counts can change over time. Moreover, these longitudinal changes in platelet contractile force were associated with the clinical bleeding score. Higher bleeding scores occurred when the patient's platelet counts were <40 K/uL and platelet forces were <25 nN, and lower bleeding scores coincided with either an increase in platelet count, platelet force, or both. These data highlight the synergistic relationship between platelet count and platelet force. Additionally, leveraging a mixed mean model, and keeping platelet count constant while adjusting for patient and visit, we found that platelet contractile force is 10.79 nN less during instances where a patient's bleeding score is between 2–4 versus when the bleeding score is either 0 or 1. Colored zones match those established in Fig. 2e. Source data are provided as a Source Data file.

demonstrated identical trends as the polyclonal ITP patient antibodies regarding antibody-integrin conformation and total contractile force (Fig. 5c). Antibodies binding and stabilizing the bent conformation decrease platelet contractile force, whereas antibodies stabilizing the extended-closed slightly decrease force, and antibodies stabilizing the extended-open conformation increase force. Taken together, the monoclonal and polyclonal antibody data suggest that integrin conformation is a key driver of single platelet force, further confirming that antibodies can bind to integrins on cells and immuno-mechanically modulate cellular biophysical behavior.

Based on the ability of monoclonal antibodies to modulate platelet contractile force up and down, we then explored the potential therapeutic application of adding force-enhancing antibodies to platelets with low contractile forces. Healthy control platelets were first treated with the most potent low force antibody MBC 290.5 for 15 min. We then added either a low concentration (0.25 μg/mL) or a high concentration (2.5 μg/mL) of one force enhancing antibody, either MBC 314.5 or Libs1. We found that average platelet contractile force significantly increased up to 154% percent with MBC 314.5 and up to 61% with Libs1 as compared to the low force antibody only condition

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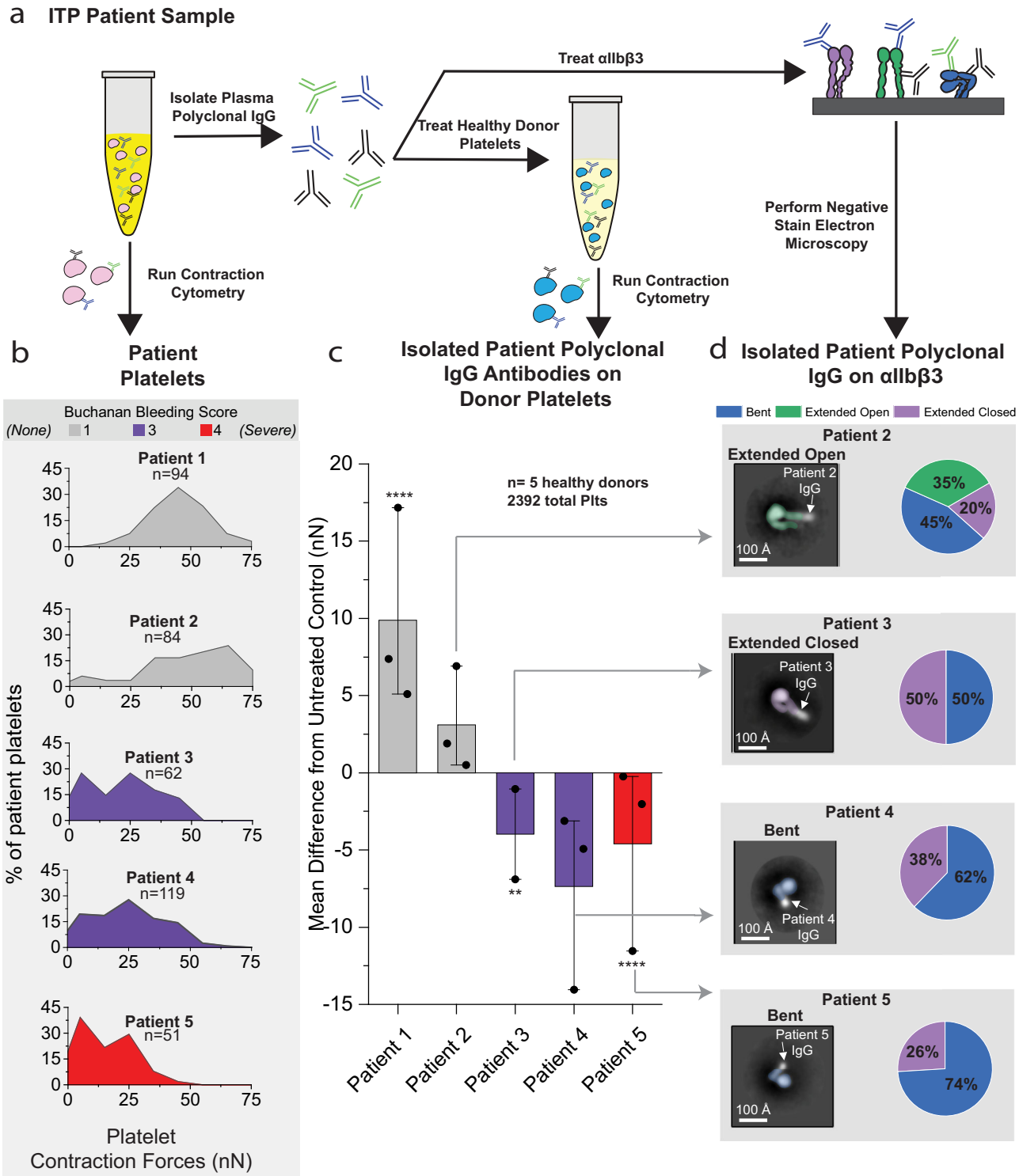

Fig. 4 | ITP Patient polyclonal IgG platelet-associated (PA) antibodies are directed towards epitopes the integrin $\alpha_{IIb}\beta_3$ and modulate platelet force in a confirmation-dependent manner. a We performed contraction cytometry on the platelets from ITP patients and extracted polyclonal IgG antibodies from the plasma for further analysis. **b** In those patients, the extent of clinical bleeding severity scores correlated with the lack of highly contractile platelets. **c** We found that isolated IgG antibodies (10 μg/mL) from those ITP patients with bleeding modulated the forces of platelets from healthy donors ($n = 3,3,2,3,3$ donors/condition with 379, 369, 338, 393, 390 antibody treated platelets, respectively and 532 untreated control platelets analyzed as shown in Supplementary Fig. 9). **d** As platelet contractile force is transmitted via the $\alpha_{IIb}\beta_3$ integrin, we used negative stain electron microscopy to study those specific antibody-integrin interactions. We found that autoantibodies from ITP patients with higher bleeding scores stabilized integrins in the bent and extended closed conformations. For (**c**), all antibody-treated platelets were compared to the untreated condition, and statistical significance was determined by mixed effects model, to account for within-subject variation, followed by the Benjamini-Hochberg's posthoc test to account for multiple comparisons. Antibody treated platelet contraction force difference for (**c**) is shown as mean ± the minimum and maximum differences from the untreated control mean. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{****}P \le 0.0001$ and specific p-values are shown in Supplementary Table 5. Source data are provided as a Source Data file.

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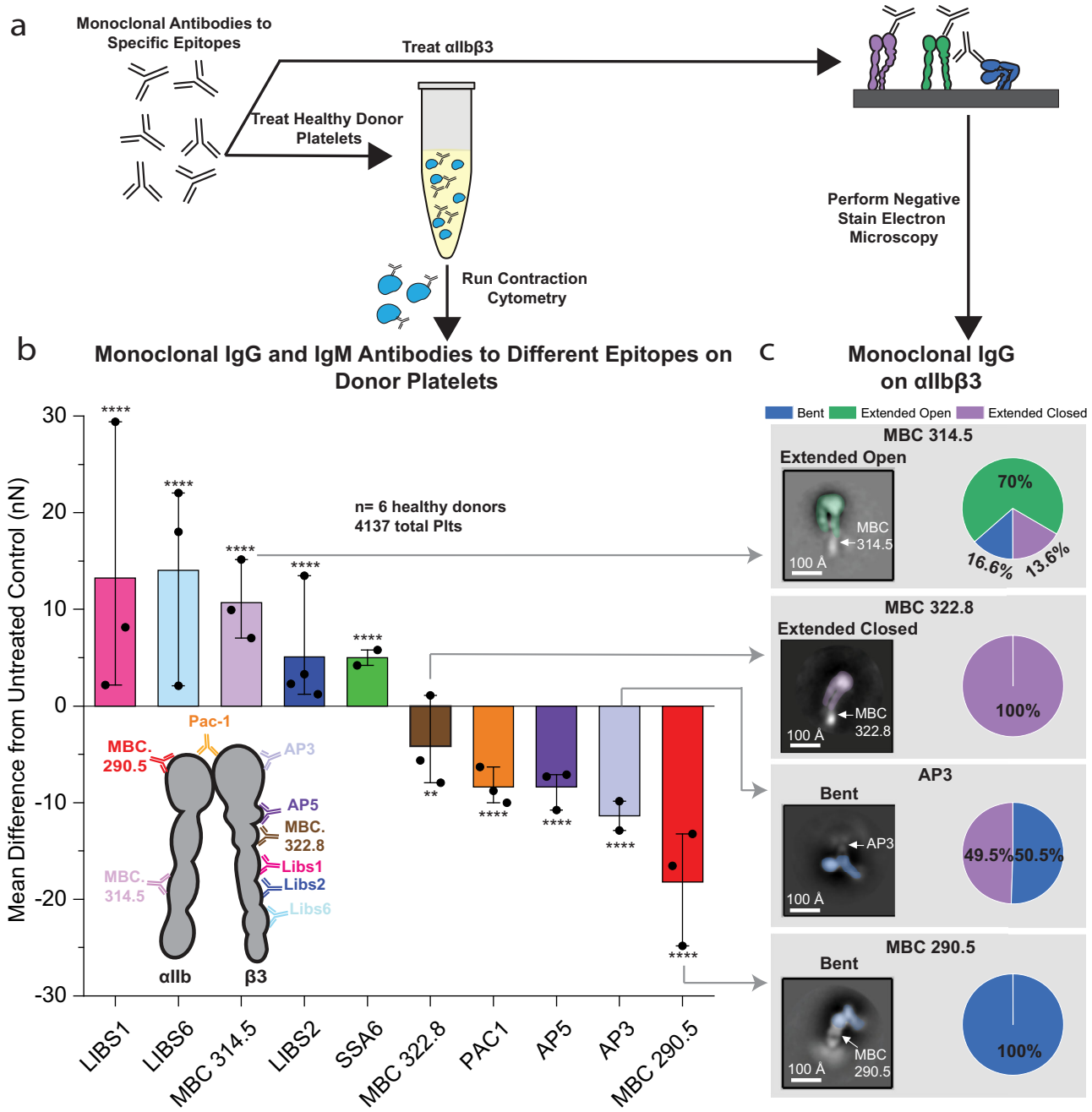

Fig. 5 | Immuno-mechanical modulation by autoantibodies directed to specific $\alpha_{IIb}\beta_3$ integrin epitopes is associated with increases and decreases in platelet contractile force. a To investigate the relationship between conformation- and epitope-dependent platelet contractile force modulation, we used a panel of well-characterized monoclonal antibodies (2.5 μg/mL) directed toward different epitopes of $\alpha_{IIb}\beta_3$. **b** Our data show that exposure to monoclonal antibodies alone can modulate average platelet force by ~ ±15 nN (55%). Moreover, a pattern became evident in which antibodies that bind closer to the tail region increase platelet contractile force, whereas those that bind toward the head region decrease force (n = 3,3,3,4,2,3,3,3,2,3 donors/condition with 434, 514, 450, 421, 213, 331, 176, 144, 123, 192 antibody treated platelets, respectively and 1139 untreated control platelets analyzed as shown in Supplementary Fig. 10). **c** Consistent with our electron microscopy data from patient polyclonal autoantibodies, negative stain electron microscopy showed that antibodies that cause a decrease in contractile force bound mostly to the bent conformation and extended closed formation of the integrin, although this data show that there is only a minor decrease in force when all integrins are stabilized in the extended closed conformation. Antibodies that stabilized integrins in an extended open conformation were associated with increased forces. For (**b**), all antibody-treated platelets were compared to the untreated condition, and statistical significance was determined by mixed effects model, to account for within-subject variation, followed by the Benjamini-Hochberg's posthoc test to account for multiple comparisons. Antibody treated platelet contraction force difference for (**b**) is shown as mean ± the minimum and maximum differences from the untreated control mean. *$P \le 0.05$, **$P \le 0.01$, ****$P \le 0$ .0001 and specific p-values are shown in Supplementary Table 5. Source data are provided as a Source Data file.

(Fig. 6 and Supplementary Table 5). Although the platelet contractile forces of the untreated control did not completely recover, our work here suggests that high force monoclonal antibodies could potentially be administered therapeutically to increase a patient's platelet contraction force, which our data shows to be associated with decreased bleeding risk.

As platelets must adhere, spread, and then contract in our system, we investigated whether these observed decreases in force correlate

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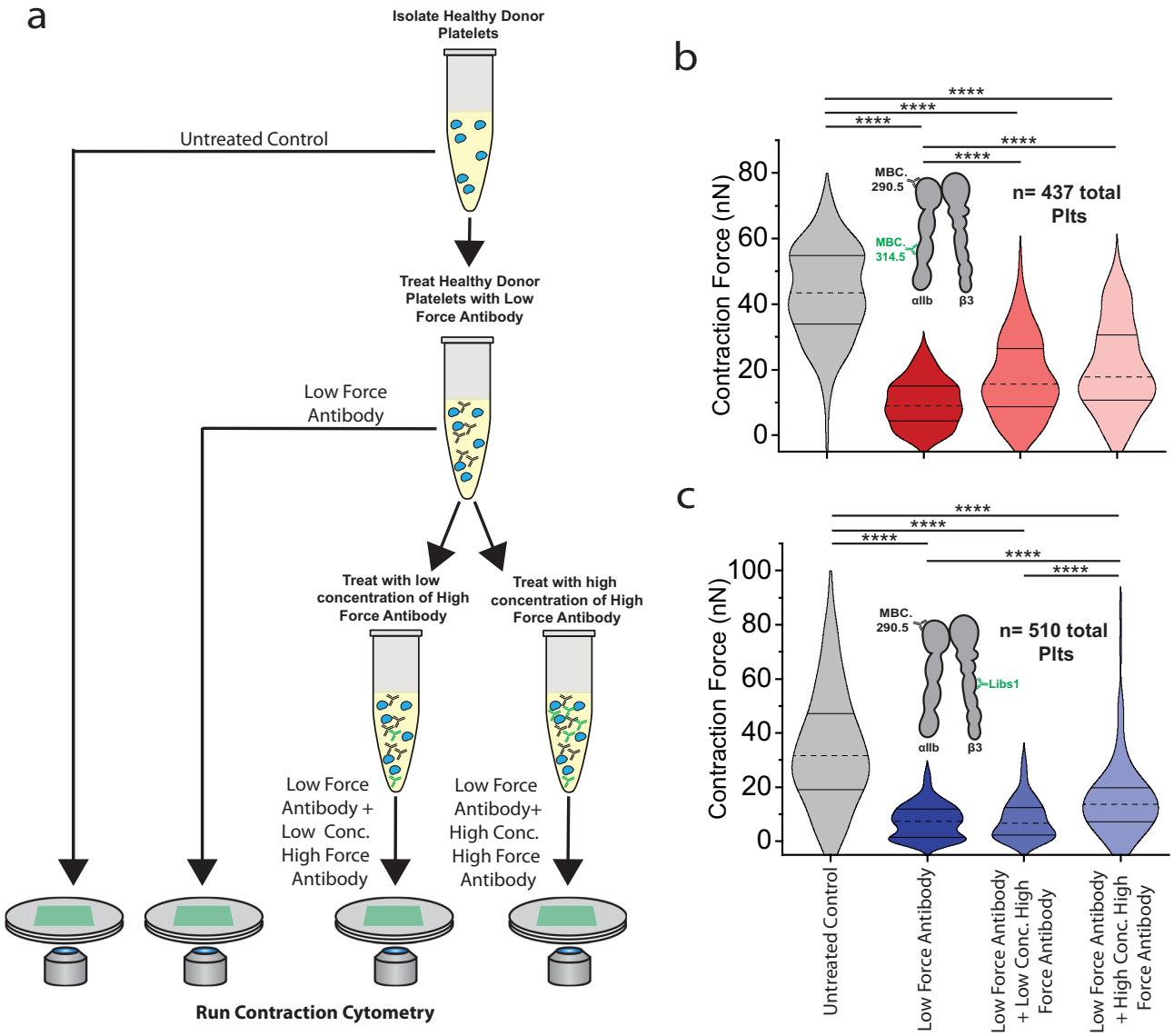

Fig. 6 | Exposure to antibodies that induce "high" platelet contractile force partially restore the contractile forces of platelets first treated with "low" contractile force antibodies. a To investigate whether high force antibodies have therapeutic potential to increase platelet forces, we measured the contraction force of healthy donor platelets (untreated control, $n = 278$ platelets). We next treated a subset of the healthy donor platelets with 2.5 μg/mL of the most potent low force antibody MBC 290.5 ($n = 235$ platelets) and measured the contractile force. Finally, we took a subset of the MBC 290.5 treated platelets and then treated with a high force antibody (Libs 1 or MBC 314.5) at a low concentration (0.25 μg/mL) or a high concentration (2.5 μg/mL). **b** MBC 314.5 significantly increased the contraction force of MBC 290.5 treated platelets at both low (+111%, $n = 107$ platelets) and high (+154%, $n = 106$ platelets) concentrations. **c** Libs 1 significantly increased the contraction force of MBC 290.5 treated platelets at a high concentration (+61%, $n = 120$ platelets) but not at a low concentration ($n = 101$ platelets). Violin plots show the median (dotted line) and the 25th and 75th percentile (solid lines) of contractile platelets. For (**b**, **c**), statistical significance was determined by mixed effects model, to account for within-subject variation, followed by the Benjamini-Hochberg's posthoc test to account for multiple comparisons. ****$P \leq 0.0001$ and specific p-values are shown in Supplementary Table 6. Source data are provided as a Source Data file.

with a concomitant decrease in platelet adhesion and spreading. To that end, we performed standard platelet adhesion and spreading assays[63] on fibrinogen-coated glass surfaces and treated healthy donor platelets with the epitope-specific monoclonal antibodies. We found that there was a moderate correlation in antibody induced changes between platelet force and adhesion, and a weak correlation between platelet force and platelet spreading (Supplemental Fig. 11). Despite the overall trend, some antibodies increased or decreased forces significantly, while having only a minor effect on adhesion. Some antibodies increased platelet force yet had a negligible effect on platelet adhesion compared to an untreated control condition (Supplemental Fig. 11). These data suggest that these integrin-dependent biophysical behaviors can be modulated by various anti-integrin antibodies including those that inhibit ligand binding functions, those that enhance ligand binding functions and those that recognize or modulate post-ligand occupancy conformational changes. Additionally, we found that antibodies that competitively bind, and therefore have closely adjacent or overlapping epitopes, can induce different changes to downstream biophysical behaviors. For example, abciximab[64], PAC-1, and the small molecule inhibitors tirofiban and eptifibatide all target

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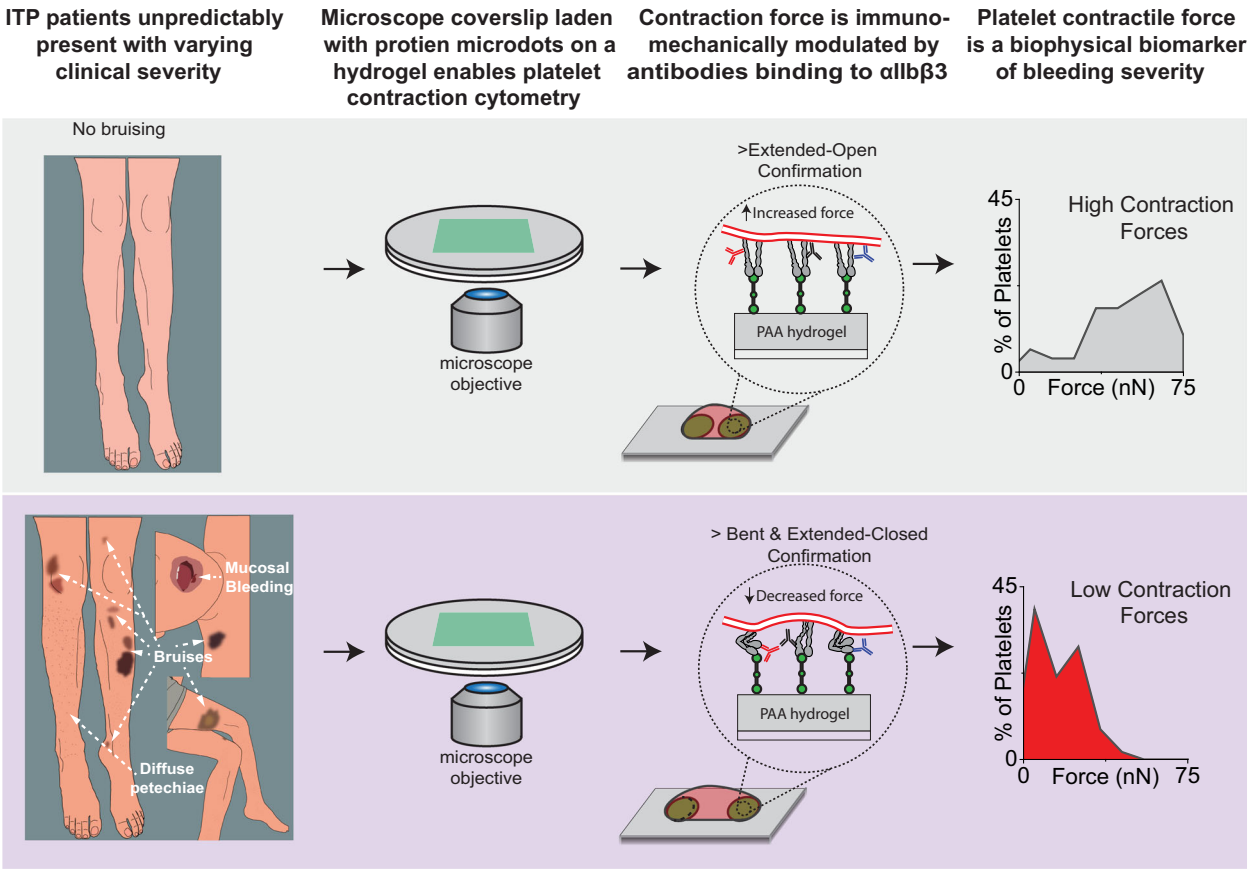

Fig. 7 | Force cytometry reveals single platelet contraction force as a potential clinical biophysical biomarker for bleeding severity modulated by auto-antibodies targeted to the αIIbβ3 platelet integrin. Our micropatterned hydrogel-laden microscope coverslip-based cellular contraction cytometry technology demonstrates a direct link between platelet biophysics and a patient's bleeding phenotype. In the case of ITP, we found that a patient's bleeding score directly correlates with a decrease in single platelet contraction. Mechanistically, we discovered that a patient's autoantibodies targeted against the αIIbβ3 integrin "immuno-mechanically" modulate platelet contractile forces (increasing or decreasing) in a conformation- and epitope-dependent manner. Antibodies that bind to the bent or extended-closed integrin conformation decrease platelet contractile force whereas antibodies that bind to the extended-open integrin conformation increase force. Therefore, platelet contractility is associated inversely with bleeding severity in that patient platelets with low contraction forces are associated with an increased risk of a bleeding phenotype, whereas patients with highly contractile platelets will likely be asymptomatic.

the binding pocket of $\alpha_{IIb}\beta_3$, however, at the concentrations used that are comparable to therapeutic plasma concentration ranges[65–67], abciximab completely blocks adhesion on our system, while PAC-1, tirofiban, and eptifibatide substantially reduced adhesion (Supplementary Figs. 12 and 13) and lowered contractile force. Similarly, 10E5 and MBC 290.5 bind to similar locations[68], yet MBC 290.5 leads to lower forces whereas 10E5 completely blocked the platelet's ability to spread to the neighboring microdot (Supplementary Figs. 12 and 13). Finally, it should be noted that other factors in addition to integrin conformation may modulate force. For example, prior work has shown that eptifibatide increases the affinity of $\alpha_{IIb}\beta_3$ and places it in the open/extended conformation (shown via PAC-1 binding)[69]. However, our results show that the forces decrease, as eptifibatide also decreases adhesion and contractile forces (Supplementary Fig. 13). As such, our work suggests that even slight difference in the epitopes bound by various antibodies may lead to moderate differences in their biophysical and modulatory effects on platelets, although further experiments would be needed to better understand this phenomenon.

Discussion

By leveraging our high-throughput contraction cytometer, which we have now simplified into a coverslip-based technology that can be adapted to standard microscopy and measuring single cell platelet forces (>10,000 measurements), we show a direct link between cellular mechanical measurements at the single cell level and patient phenotype in ITP. Clinical biomarkers are typically biological or biochemical in nature, and here we introduce a potential type of clinical biomarker, one that is biophysical in nature, as we specifically demonstrate how decreased platelet contractile forces are associated with bleeding in the setting of an autoimmune disease. As such, our work sets the stage for a paradigm of diagnostic and clinical assay, where single cell biophysics could be utilized to predict clinical outcomes. Additionally, because our system allows for single cell measurements of force at high throughput, we were able to investigate the mechanistic underpinnings of decreased platelet force in ITP and describe the cause and effects of how decreased platelet contraction may lead to bleeding in ITP (Fig. 7).

Moreover, our study links integrin conformation to a specific clinical pathology, where a patient's own immune system by way of antibodies can modulate the biophysical behavior of their cells causing a clinical phenotype depending on the integrin confirmation and epitope the antibodies bind. This research also points to a possible protective effect from the autoantibodies, where increased platelet strength counters the low platelet count. Such an approach may offer an innovative therapeutic strategy. Additionally, one need for future work is a better understanding of how antibodies to varying epitopes cause the high variation in downstream forces. We speculate that the varying antibody binding locations modulate integrin conformational flexibility, which has recently been tied to single cell forces[70].

More broadly, this concept of "immuno-mechanical modulation" will likely have important implications for the scientific field as a whole and potentially lead to different therapeutic approaches, as we show that single platelet force can be "recovered" by leveraging force enhancing monoclonal antibodies. Our work questions the canonical characterizations of antibodies as either "inhibitory" or "stimulatory" and instead suggests that biophysical function can be modulated on a much finer scale. This more precise control is important since integrin-mediated mechanotransduction affects numerous cellular functions such as motility, proliferation, differentiation, cytoskeletal dynamics, gene expression, and overall cellular homeostasis. Because monoclonal antibodies can be easily screened and manufactured, one can also envision therapeutically administering monoclonal antibodies to specifically modulate cellular mechanical phenomena, such as biophysically controlling stem cell differentiation in vivo. In addition, as autoantibodies directed towards integrins[40] and proteins are implicated in numerous viral infections, such as in Dengue[40] and most recently in SARS-CoV-2[71,72], as well as immune disorders such as systemic lupus erythematosus, rheumatoid arthritis, diabetic retinopathy, and scleroderma, our research highlights a need to test how such antibodies affect underlying cell and protein mechanical function.

Methods

Platelet preparation

Written and informed consent for healthy donor platelets was obtained according to Georgia Tech Institutional Review Board H15258. Similarly, for ITP patient samples, blood was drawn after written and informed consent according to Emory University Institutional Review Board 00103528. Blood was drawn into acid-citrate-dextrose (ACD) solution 2 (VWR) and was subsequently centrifuged at 150 G for 15 min without brake and the resulting platelet rich plasma (PRP) was centrifuged again with an additional 10% ACD by volume at 900 G for 5 min without brake. The supernatant, platelet poor plasma, was discarded or utilized to isolate patient polyclonal IgG antibodies. The platelet pellet was resuspended into HEPES modified tyrodes buffer and was gel filtered into this same buffer using a sepharose (Sigma) gel filtration column. Platelets were then diluted to 2 million/mL prior to contraction cytometry to reduce paracrine signaling and minimize platelet aggregate formation.

Buchanan bleeding scores, platelet count, and mean platelet volume

Following consent for ITP patients, one of two hematologists that are blinded to the platelet contraction results assessed Buchanan bleeding scores of each patient. Investigators were blinded to bleeding scores prior to platelet contraction cytometry, but not blinded to platelet count and Mean Platelet Volume, which were provided with receipt of patient blood sample. No statistical method was used to predetermine sample size, and no data were excluded from analyses. To capture as many patient samples as possible, all samples, regardless of self-reported sex were included in this study. Our end study included 23 male and 30 female patients. No significant sex differences were observed. The source data includes identification of sex of each sample.

Platelet contraction cytometer device fabrication

Plasma treated circular coverslips (25 mm Circle No.1) were incubated in a 10% (3-Aminopropyl) trimethoxysilane (Sigma Aldrich), 90% Ethanol, 0.01% Glacial acetic acid solution for 90 min at 60 °C. The coverslip was then rinsed 3 times with 70% ethanol and then rinsed 3 times with DI water. The coverslips were then treated with a 2% glutaraldehyde solution for 30 min at room temperature. After treatment, coverslips were rinsed with DI water and dried with Nitrogen gas.

Alexa Fluor 488 tagged fibrinogen was incubated on square (10 mm × 10 mm × 3 mm) polydimethylsiloxane (PDMS) at 30 μg/mL for 30 min at 37 °C before being rinsed with water and dried with nitrogen gas. These fibrinogen coated PDMS squares were then placed on to a silicon mold and lifted off to create a micropatterned PDMS "stamp" (The silicon mold was created using standard etching and lithography techniques). Then the newly made PDMS stamp was then placed on to a plasma treated circular coverslip (25 mm Circle No.1) and then lifted off to transfer the microdot pattern to the coverslip.

The assembly of the platelet contraction cytometer was performed in a nitrogen filled glovebox. The glutaraldehyde treated coverslip and micropatterned coverslip were placed in the glove box antechamber and vacuumed prior to assembly in the glove box. Premixed polyacrylamide hydrogels were mixed with N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Sigma Aldrich), ammonium persulfate (Sigma Aldrich), and acrylic acid N-hydroxysuccinimide ester (NHS-ester) (Sigma Aldrich) and 40 μL of this hydrogel solution was pipetted onto each glutaraldehyde treated coverslip (A glove box isn't necessary, but it helps speed up the polymerization and allows for lower concentrations of APS, TEMED, and NHS-Ester to be used). The micropatterned coverslip was then inverted onto the hydrogel solution immediately after, creating a "sandwich" and the hydrogel was allowed to polymerize in the glovebox for 90 min to ensure complete polymerization. After polymerization, the hydrogel "sandwich" was removed from the glove box and the top micropatterned coverslip was removed and discarded as the micropattern is now transferred to the top of the formed hydrogel, this creating the contraction cytometer. The newly formed contraction cytometers were stored for up to 7 days in PBS at 4 °C in petri dishes.

Platelet contraction cytometer device operation

Each contraction cytometer contains large arrays of fibrinogen microdot pairs that are patterned onto the surface of a polyacrylamide hydrogel with a stiffness of 75 kPa. Each microdot pair possesses two microdots with a radius of 0.8 μm and a separation of 4 μm. These values for microdot size and spacing ensure that platelets preferentially attach to the microdots and are able to spread to neighboring microdot in the microdot pair and contract. Once fabricated, 2 million/mL platelets in 3-mM $CaCl_2$, 3-mM $MgCl_2$, and 1-U/mL thrombin are plated onto the contraction cytometer for 90 min and allowed to displace the microdot pairs. Similar to a spring, the fibrinogen microdot displacement is proportional to the contraction force.

Platelet contraction was imaged on a Zeiss LSM 780/ELYRA PS1 confocal microscope using a 20x/0.8NA Plan Apochromat lens. Platelets were tagged with cell mask orange 554/567 nm (Thermo Fisher Scientific). Images were analyzed using a MATLAB script which calculated the center-to-center distance between fibrinogen microdots. The microdot displacement produced by each contracting platelet was compared to an uncontracted reference dot pair. Only platelets that have adhered and spread across microdot pairs are analyzed for contractile force. Platelets are only adhered to the green fibrinogen dots and not the polyacrylamide hydrogel (black). Supplementary Fig. 1 shows an example where majority of platelets on the surface adhere to a microdot, span across to the neighboring microdot and contract while Supplementary Fig. 11 shows examples of platelets that are poorly attached and not able to contract the microdots together. The script for analysis of contracting platelets is freely available at GitHub (https://github.com/davidrmyers/platelet-contraction). Traction forces (T) of individual platelets was calculated as shown in Eq. 1:

$$T = \frac{2\pi G a\left(x_s - x_f\right)}{2 - \upsilon} \tag{1}$$

where G is the shear modulus, a is the microdot radius, υ is Poisson's ratio, xs is the starting distance, and xf is the final distance post contraction.

Like prior work in this area[23,73–77], our force measurements presume that platelets adhere only to the fibrinogen microdots and not to the bare polyacrylamide in between and around the microdots. Prior work has established that polyacrylamide is protein repellant and does not natively support cell adhesion without modification[78], or when a non-adhesive protein is micropatterned on a polyacrylamide surface[79]. Our own data supports this idea as platelets do not adhere to the blank polyacrylamide and are consistently co-located with fibrinogen. As activated platelets randomly land on the contraction cytometer surface they come into contact with blank polyacrylamide and fibrinogen. However, as platelets are not found only on the blank polyacrylamide regions and are only co-located with fibrinogen, this supports the idea that platelets do not adhere to polyacrylamide. However, we acknowledge that it would be impossible to completely rule out some platelet adhesion to bare polyacrylamide. Other cells that extend filipodia and lamellipodia past ECM micropatterns and into bare polyacrylamide regions have been shown to have zero adhesion, but unlike platelets, these cells remain living and dynamically modulate these structures[79–83]. As activated platelets are at the end stages of their life, it is not possible to perform this experiment.

Patient polyclonal IgG isolation and contraction experiments

13 ITP PPP samples with varying bleeding scores were sent to Versiti to undergo platelet antibody screen (order code: 5543). All positive samples had platelet reactive IgG antibodies and not IgM. As such, IgG antibodies from all positive patient samples with varying bleeding scores were isolated using Nab Protein G Spin Columns (Thermo fisher Scientific). Once isolated, healthy donor platelets were treated with 10 μg/mL of patient polyclonal IgG antibodies for 15 min prior to platelet activation with thrombin and incubation on contraction cytometer. All antibody treated conditions were run in conjunction with and compared to an untreated control.

Monoclonal antibody and small molecule platelet inhibitor contraction experiments

Healthy donor platelets were treated with 2.5 μg/mL of well characterized monoclonal antibodies or the small molecule platelet inhibitors tirofiban or eptifibatide for 15 min prior to platelet activation with thrombin and incubation on contraction cytometer. All antibody treated conditions were run in conjunction with and compared to an untreated control.

MBC 290.5, AP3, AP5, MBC 322.8, MBC 314.5 were purchased from Kerafast. PAC-1 was purchased from Fisher scientific. LIBS2 was purchased from Sigma Aldrich. Abciximab and 10e5 were purchased from Absolute Antibody. HIP8 was purchased from Abcam. LIBS1 and LIBS6 were a gift from Mark Ginsberg's lab. SSA6 was a gift from Joel Bennett's lab. Eptifibatide and Tirofiban were purchased from Cayman.

Monoclonal antibody negative stain electron microscopy

Recombinant human Integrin $\alpha_{IIb}\beta_3$ (R&D biosystem) complexed with monoclonal antibody was diluted to 0.04 mg/ml in PBS before grid preparation. A 3 μL drop of diluted protein was applied to previously glow-discharged, carbon-coated grids for ~60 s, blotted and washed twice with water, stained with 0.75% uranyl formate, blotted, and air-dried. Between 50 and 70 images were collected on a Talos L120C microscope (Thermo Fisher) at 73,000 magnification and 1.97 Å pixel size. Relion-3.1 was used for particle picking and 2D classification.

Patient polyclonal antibody and integrin $\alpha_{IIb}\beta_3$ complex preparation and purification

First to prepare the Integrin $\alpha_{IIb}\beta_3$ protein polyclonal antibody complex, we purified patient polyclonal IgG using Protein-A/ Capture Select-XL resin (Thermo Fisher). Purified IgG was subjected to Papain mediated proteolytic digestion for Fab fragmentation. Fab was further purified away from undigested IgG and Fc by incubation with Protein-A agarose beads. Purified Fab was incubated with Integrin $\alpha_{IIb}\beta_3$, at molar ratio of 20:1, followed by purification of Fab-bound antigen complexes using size exclusion chromatography. Fractions corresponding to Integrin $\alpha_{IIb}\beta_3$protein polyclonal antibody complex was used for subsequent sample preparation, Imaging and data analysis as mentioned in the above section by Negative stain-EM.

Platelet force recovery with force enhancing monoclonal antibodies

To investigate whether force enhancing antibodies have therapeutic potential to increase platelet forces, we measured the contraction force of healthy donor platelets, we then treated a subset of the healthy donor platelets to 2.5 μg/mL of MBC 290.5 (low platelet force creating antibody) for 15 min and then performed contraction cytometry. Finally, we took a subset of the MBC 290.5 treated platelets and then treated with a high force antibody (Libs 1 or MBC 314.5) at a low concentration (0.25 μg) or a high concentration (2.5 μg) and again measured the force using contraction cytometry.

Platelet adhesion assays with monoclonal antibodies

Platelets were diluted to 10×10^9/L to ensure the measurement of single platelets and then incubated with selected monoclonal antibody for 15 min prior to experimentation. Antibody treated platelets were then incubated on coverslips on 100 μg/mL human fibrinogen (Enzyme Research Laboratory) and allowed to adhere for 2 h. Platelets were imaged with an Eclipse Ti2 inverted microscope using a 40x/1.30NA Plan Fluor lens and then analyzed for adhesion density and spreading area using a software our lab developed, iClots (https://www.iclots.org/software)[84].

Statistical and data analysis

Tukey's adjusted one-way ANOVA was used to compare single platelet forces of patients possessing various bleeding scores and to compare MPV and platelet counts of patients with various bleeding scores. Post hoc multiple comparisons of means was conducted using the Scheffe Test to maintain a 95% family-wise confidence level. For experiments involving monoclonal and polyclonal antibodies, single platelet force was assessed with mixed effects model to account for within subject correlation (fixed and random effects), followed by the Benjamini-Hochberg's posthoc test to account for multiple comparisons. We chose a mixed effects model because it has a better statistical fit and matches the study design better than a traditional regression model. Intraclass correlation coefficients (ICCs) were calculated to determine whether a mixed model was necessary. Our ICC was 0.22 and due to our ICC > 0, there was sufficient evidence to indicate that modeling the platelets within person was the most efficient approach. As such, we utilize the mixed effects model and analyze the data at the platelet level, clustered by donor. In this case, the model uses hundreds of data points per donor to estimate the within-donor correlation and takes into consideration both the fixed effects (shared across the donors) and the random effects (specific to each donor) in order to generate a *p*-value. The model treats each donor's data points as not independent, but as repeated measures.

For the ROC analysis, we utilized a multivariable logistic model to test the association of platelet force, MPV, and platelet count to our endpoint of bleeding score (0–1 vs 2–4). We also considered bleeding score of 0–2 vs 3–4 secondarily. The standard error of the AUC curve was computed by using a commonly used method for comparing AUC[85] and significance was based on the z-distribution. Origin 2022b was used for data plotting of all graphs in manuscript. Statistical analysis was performed using R 4.2.1 (https://www.R-project.org/).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The numerical data necessary to recreate main text figures, Supplementary figures and the raw platelet contraction data for each experimental group are provided in the source data file. Patient demographics are provided in the supplementary information. Platelet contraction images are available from the corresponding authors David R. Myers and Wilber A. Lam upon request within 5 business days. All data will be de-identified. Source data are provided with this paper.

Code availability

The MATLAB script for measuring single platelet contraction is freely available at GitHub (https://github.com/davidrmyers/platelet-contraction). The software utilized for adhesion analyses is freely available at iClots (https://www.iclots.org/software)[84].

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Competing interests
The authors declare no competing interests.

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