Article

Antibody epitopes in vaccine-induced immune thrombotic thrombocytopaenia

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Vaccine-induced immune thrombotic thrombocytopaenia (VITT) is a rare adverse effect of COVID-19 adenoviral vector vaccines[1–3]. VITT resembles heparin-induced thrombocytopaenia (HIT) in that it is associated with platelet-activating antibodies against platelet factor 4 (PF4)[4]; however, patients with VITT develop thrombocytopaenia and thrombosis without exposure to heparin. Here we sought to determine the binding site on PF4 of antibodies from patients with VITT. Using alanine-scanning mutagenesis[5], we found that the binding of anti-PF4 antibodies from patients with VITT ($n = 5$) was restricted to eight surface amino acids on PF4, all of which were located within the heparin-binding site, and that the binding was inhibited by heparin. By contrast, antibodies from patients with HIT ($n = 10$) bound to amino acids that corresponded to two different sites on PF4. Biolayer interferometry experiments also revealed that VITT anti-PF4 antibodies had a stronger binding response to PF4 and PF4–heparin complexes than did HIT anti-PF4 antibodies, albeit with similar dissociation rates. Our data indicate that VITT antibodies can mimic the effect of heparin by binding to a similar site on PF4; this allows PF4 tetramers to cluster and form immune complexes, which in turn causes Fcγ receptor IIa (FcγRIIa; also known as CD32a)-dependent platelet activation. These results provide an explanation for VITT-antibody-induced platelet activation that could contribute to thrombosis.

VITT is a rare but serious adverse effect of adenoviral vector vaccines against SARS-CoV-2. The clinical picture of VITT is moderate to severe thrombocytopaenia together with arterial and/or venous thrombi, often occurring in unusual locations[1–3]. These findings resemble the immunological drug reaction HIT, which presents clinically as thrombocytopaenia and thrombosis in patients who have previously been exposed to heparin[4]. VITT most closely resembles the exceptionally rare spontaneous HIT, which occurs in the absence of heparin[6,7].

HIT is caused by immunoglobulin G (IgG) antibodies that bind to neoepitopes on PF4 (also known as CXCL4), a 70-amino-acid cationic protein that is contained within platelets[8,9]. The neoepitopes become exposed after heparin, a large anionic polysaccharide, binds to a specific site on PF4, which causes tetramers to cluster together. The IgG-specific antibodies bind to PF4–heparin to form immune complexes, which activate platelets through FcγRIIa receptors, leading to an intense activation of platelets and the release of procoagulant-rich microparticles[10]. Other cells, including monocytes, are also activated by these immune complexes, which amplifies the hypercoagulable state in patients with HIT[11]. It has been postulated that VITT has a similar pathophysiology to HIT, and several studies have shown that high levels of anti-PF4 antibodies are present in samples from patients with VITT[1–3,12]. However, VITT is a unique syndrome as it occurs without heparin exposure, and the pattern of platelet reactivity in vitro does not exhibit the typical heparin dependence that is seen with HIT.

In this report, we describe the binding site and characteristics of anti-PF4 antibodies that developed in patients with VITT in response to vaccination against COVID-19 with an adenoviral vector. We found that patients with VITT had anti-PF4 antibodies that bound to a highly restricted site on PF4 that corresponds to the heparin-binding site. These antibodies can form platelet-activating immune complexes without heparin, potentially causing the thrombocytopaenia and clotting that are observed in VITT.

Patient demographics

Samples from patients with VITT ($n = 5$) were referred to the McMaster Platelet Immunology Laboratory for diagnosis. All patients with VITT had received a single dose of the ChAdOx1 nCoV-19 vaccine (AstraZeneca COVID-19 vaccine, AstraZeneca; COVISHIELD, Verity Pharmaceuticals and Serum Institute of India) and subsequently developed thrombocytopaenia and thrombosis; the mean age of patients was 44 years (range, 35–72 years) and 2 out of 5 (40%) were female. The time from first dose of the ChAdOx1 nCoV-19 vaccine to sample collection was 14–40 days (mean, 28 days). All samples from patients with VITT (hereafter, VITT samples) had antibodies against PF4 (mean optical density (OD), 2.71; range, 0.763–3.347).

The samples from patients with VITT were compared to samples from patients with HIT ($n = 10$) who had thrombocytopaenia after receiving heparin and had a high clinical probability score (4Ts score of at least 4), with detectable anti-PF4–heparin antibodies and evidence of platelet activation in vitro. The mean age of patients with HIT was 69 years (range, 52–81 years) and 5 out of 10 (50%) were female. Nine

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of 10 (90%) patients with HIT experienced thrombosis. The time from heparin initiation to sample collection was 6–27 days (mean, 14.3 days). All samples from patients with HIT (hereafter, HIT samples) had detectable anti-PF4–heparin antibodies (mean OD, 3.10; range, 2.329–3.897).

Platelet activation profiles of VITT antibodies

Using a functional platelet activation assay (serotonin release assay; SRA), we showed that VITT and HIT samples had unique patterns of platelet reactivity in vitro. VITT samples did not exhibit any dependence on heparin for platelet activation in vitro (Extended Data Fig. 1a). All VITT samples showed strong, dose-dependent PF4-mediated platelet activation with 50 μg ml^{-1} PF4 (Extended Data Fig. 1b). Complete inhibition of platelet activation by VITT and HIT samples was achieved with the addition of IV.3, an FcγRIIa-blocking monoclonal antibody[4]. These results indicate that VITT antibodies require PF4 for platelet activation, and that this is mediated by the engagement of FcγRIIa receptors, consistent with the effects of an immune complex. By contrast, HIT samples activated platelets with two distinct profiles. Patients with HIT with heparin-dependent antibodies were patients who were exposed to heparin and whose antibodies activated platelets in the SRA in the presence of heparin (Extended Data Fig. 1c). Patients with HIT with heparin-independent antibodies were patients who were exposed to heparin and whose antibodies activated platelets in the SRA both in the presence and in the absence of heparin (Extended Data Fig. 1c). All HIT samples also activated platelets with the addition of PF4 (Extended Data Fig. 1d).

Binding site of VITT antibodies on PF4

Previous studies have shown that the binding epitopes of HIT antibodies are non-contiguous and conformation-dependent[13]. Therefore, to identify the specific amino acid targets of VITT antibodies on PF4, we used alanine-scanning mutagenesis and produced 70 unique recombinant PF4 mutants, each differing by a single amino acid[5]. We defined a critical binding amino acid as one that when mutated caused a reduction in binding of more than 50% in the corresponding PF4 mutant compared to wild-type PF4. We identified eight surface amino acids that were necessary for binding of VITT samples (Arg22, His23, Glu28, Lys46, Asn47, Lys50, Lys62 and Lys66; Table 1, Fig. 1a). The PF4 mutants R22A and E28A affected the binding for all five VITT samples. This restricted epitope is consistent with limited B cell clonality and suggests that the binding site of VITT antibodies is to a specific site on PF4. We observed that four of the eight amino acids that comprised the VITT epitope (Arg22, His23, Lys46 and Lys66) corresponded to amino acids on PF4 to which heparin binds[14] (Fig. 1b). We postulate that binding of VITT anti-PF4 antibodies to the heparin-binding site on the PF4 tetramer explains why the addition of heparin in vitro does not augment platelet activation, as seen in HIT; rather, it inhibits platelet activation, presumably by displacing VITT anti-PF4 antibodies. Furthermore, in a PF4 enzyme immunoassay (EIA), we showed that the binding of VITT antibodies to PF4 was inhibited by therapeutic concentrations of heparin in four of the available VITT samples (Extended Data Fig. 2). Our results explain why some VITT samples tested in previous studies[1,2] were inhibited by therapeutic doses of heparin. These findings suggest that VITT antibodies cause platelet activation through a heparin-like mechanism by stabilizing complexes of PF4, aligning the Fc regions of the VITT antibodies to be in close proximity to one another and cross-link FcγRIIa receptors on platelets—similar to the mechanism of the monoclonal antibody 1E12, which can activate platelets independent of heparin[15].

The binding site of VITT antibodies on PF4 was then compared with that of HIT antibodies. When all of the critical amino acids that were identified through screening of the 10 patients with HIT were combined, there was a total of 10 amino acids (Leu8, Cys10, Cys12, Thr16, Arg22, Gln40, Asn47, Cys52, Leu53, Asp54, Lys61, Lys66 and Leu67; Table 1) that were part of the HIT epitopes, in different combinations. No single common amino acid was critical for the binding of all 10 HIT samples, probably owing to the polyclonal nature of the antibodies[5]. The loss of binding in PF4 mutants L8A, C10A, C12A, T16A, C52A, D54A and K61A corresponded to the most common amino acids that affect the binding of HIT antibodies and were common among six of the HIT samples. When displayed on the PF4 tetramer, we identified one site on PF4 that all HIT samples (10 out of 10) targeted (Fig. 1c, d). In addition, 6 of the 10 HIT samples targeted an additional site (Fig. 1d) similar to the VITT site that was within the heparin-binding site. Unlike the VITT samples, none of the HIT samples were restricted to the heparin-binding site. This is consistent with previous observations that some HIT samples contain two types of platelet-activating anti-PF4–heparin antibodies[16]. The HIT antibodies bound to a similar site as KKO, a monoclonal antibody against PF4–heparin complexes, thus explaining why HIT antibodies, but not VITT antibodies, require heparin to cross-link PF4 tetramers[5].

In addition to clarifying the binding site of VITT antibodies, these results provide an explanation for why some rapid HIT immunoassays may yield false-negative results for VITT[17]. One rapid HIT immunoassay, the latex immunoturbidimetric assay (HemosIL HIT-Ab$_{(PF4\text{-}H)}$), uses KKO to aggregate complexes of PF4–heparin. As all HIT samples have antibodies that bind to the same site as KKO, the HIT samples compete with KKO in binding the PF4–heparin complexes. By contrast, VITT antibodies bind to a different site on PF4 than KKO, and thus do not compete for binding.

Table 1 | Key amino acids in PF4 for the binding of VITT and HIT antibodies

Critical PF4 amino acids for VITT antibodies			
PF4 mutant	**Mean loss of binding compared to wild-type PF4 (%)**	**Range (%)**	**Number of VITT samples affected**
R22A	79.9	51.8–91.7	5/5
H23A	68.0	26.7–77.5	3/5
E28A	68.5	48.4–88.1	5/5
K46A	72.6	17.5–84.2	4/5
N47A	54.3	31.6–66.4	5/5
K50A	82.2	11.6–90.5	4/5
K62A	58.8	15.3–77.3	3/5
K66A	54.1	2.2–88.8	4/5
Critical PF4 amino acids for HIT antibodies			
PF4 mutant	**Mean loss of binding compared to wild-type PF4 (%)**	**Range (%)**	**Number of HIT samples affected**
L8A	48.6	12.4–76.0	6/10
C10A	49.4	5.0–73.3	6/10
C12A	45.7	4.8–74.7	7/10
T16A	41.1	4.0–78.4	6/10
R22A	36.2	1.3–63.0	5/10
Q40A	35.5	2.2–75.5	3/10
N47A	31.4	3.9–80.1	3/10
C52A	55.8	25.0–73.3	8/10
L53A	51.3	36.1–74.8	5/10
D54A	47.1	0.8–76.0	6/10
K61A	47.4	1.7–82.2	6/10
K66A	40.4	5.1–89.6	5/10
L67A	48.5	6.4–85.8	5/10

Amino acids were identified by alanine-scanning mutagenesis. n = 5 VITT samples; n = 10 HIT samples.

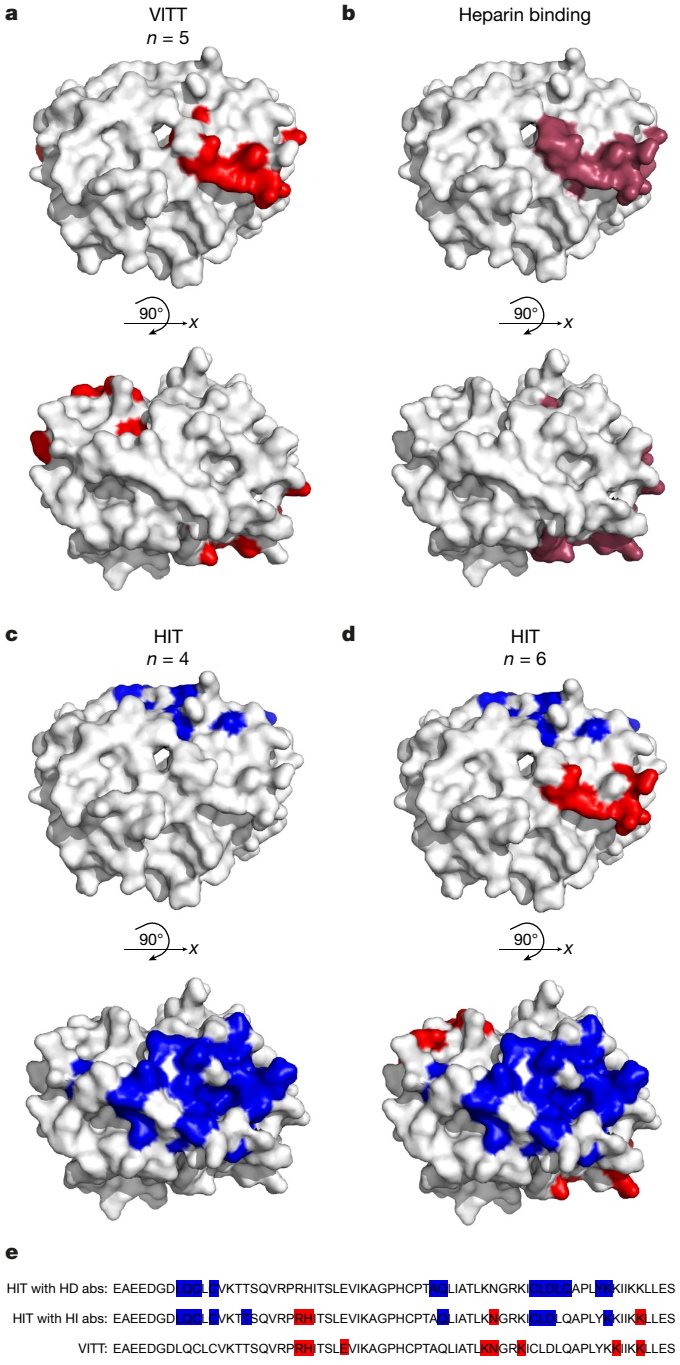

Fig. 1 | Identification of amino acids in PF4 that are critical for the binding of antibodies using alanine-scanning mutagenesis. a, **b**, For VITT samples ($n = 5$), the binding site (coloured in red; **a**) aligns within the heparin-binding site on PF4 (coloured in brown; **b**). **c**, For HIT samples ($n = 10$), one main binding site was identified for all samples (coloured in blue). **d**, An additional binding site was found in 6 out of 10 (60%) of the HIT samples (coloured in red), which aligns within the heparin-binding site on PF4. **e**, The amino acids that are predicted to be part of the binding sites for VITT and heparin-dependent (HD) and heparin-independent (HI) antibodies (abs) in HIT are highlighted on the primary sequence of PF4. Images are modified from the Protein Data Bank (PDB) entry 1RHP.

Binding kinetics of VITT antibodies

The binding responses of VITT samples ($n = 5$), HIT samples ($n = 10$) and samples from healthy control individuals ($n = 10$) to PF4 and PF4–heparin were tested using bio-layer interferometry (BLI; Fig. 2). Binding responses are a measure of the antigen-specific antibodies present in a given sample. When healthy control samples ($n = 10$) were tested with immobilized PF4 and PF4–heparin, the mean binding response (measured as the average wavelength shift (in nm) ± 2 standard deviations (2 s.d.)) was 0.0059 ± 0.11 nm and 0.031 ± 0.11 nm, respectively. All VITT ($n = 5$) and HIT ($n = 10$) samples were above the mean + 2 s.d. cut-off of 0.12 nm with immobilized PF4 and 0.14 nm with immobilized PF4–heparin, indicative of a positive binding result. The mean binding response (nm shift ± s.d.) was 1.82 ± 0.88 nm for VITT samples and 0.82 ± 0.72 nm for HIT samples ($P < 0.05$) with immobilized PF4. Similarly, the mean binding response was 1.24 ± 0.70 nm for VITT samples and 0.62 ± 0.45 nm for HIT samples ($P < 0.05$) with PF4–heparin complexes. Therefore, the binding response in VITT samples was significantly higher than that in HIT samples and healthy control samples for both PF4 and PF4–heparin, indicating a stronger antibody response in patients with VITT.

We further compared the binding response for the VITT samples to the two groups of HIT samples, with heparin-dependent ($n = 4$) and heparin-independent ($n = 6$) antibodies. The mean binding response for patients with HIT with heparin-dependent antibodies was 0.29 ± 0.18 nm, and for patients with HIT with heparin-independent antibodies the mean binding response was 1.18 ± 0.73 nm against immobilized PF4 (Fig. 2c). When samples were tested against immobilized PF4–heparin complexes, the mean binding response was 0.30 ± 0.09 nm for patients with HIT with heparin-dependent antibodies and 0.83 ± 0.48 nm for patients with HIT with heparin-independent antibodies (Fig. 2d). The binding response of VITT samples was significantly higher than that of samples from patients with HIT with heparin-dependent antibodies and healthy control samples for both PF4 and PF4–heparin (VITT versus HIT with heparin-dependent antibodies: $P < 0.01$; VITT versus healthy controls: $P < 0.001$). By contrast, the binding response of VITT samples was similar to that of samples from patients with HIT with heparin-independent antibodies. VITT samples also had a significantly higher binding response to PF4 when compared to PF4–heparin ($P < 0.05$), consistent with an inhibitory effect of heparin on the binding of VITT antibodies to PF4 (Extended Data Fig. 2).

To confirm that the binding responses in samples were due to anti-PF4 antibodies and not other serum factors, total IgG antibodies were purified from two VITT samples and then tested for their ability to bind PF4 and PF4–heparin. However, owing to sample constraints, further purification of specific VITT anti-PF4 antibodies was not performed, which represents a potential limitation for this study. The binding responses of total IgG antibodies isolated from both VITT samples against PF4 and PF4–heparin were similar to the binding responses observed with their respective sera, indicating that the binding responses are due to the anti-PF4 antibodies (Extended Data Fig. 3). In addition, two VITT, one HIT and two healthy control samples were re-tested in separate experiments and showed reproducibility (Extended Data Fig. 4).

When polyclonal antibodies interact with an antigen, the dissociation rate, which is concentration-independent, can be measured. The mean dissociation rate (k_{off} s^{-1} ± s.d.) was $6.32 \times 10^{-3} \pm 0.0077$ s^{-1} for VITT samples, $2.34 \times 10^{-3} \pm 0.0041$ s^{-1} for HIT samples with heparin-dependent antibodies and $9.65 \times 10^{-4} \pm 0.0016$ s^{-1} for HIT samples with heparin-dependent antibodies, for immobilized PF4 (Fig. 2e). Similarly, the mean dissociation rate (k_{off} s^{-1} ± s.d.) was $3.47 \times 10^{-3} \pm 0.0029$ s^{-1} for VITT samples, $4.56 \times 10^{-4} \pm 0.0002$ s^{-1} for HIT samples with heparin-dependent antibodies and $1.07 \times 10^{-3} \pm 0.0018$ s^{-1} for HIT samples with heparin-independent antibodies, for immobilized PF4–heparin (Fig. 2f). There was no statistically significant difference in the dissociation rates of the VITT group and the two HIT groups with both PF4 and PF4–heparin (PF4: VITT versus HIT with heparin-dependent antibodies, $P = 0.482$; VITT versus HIT with heparin-independent antibodies, $P = 0.220$; PF4–heparin: VITT versus HIT with heparin-dependent antibodies, $P = 0.104$; VITT versus HIT

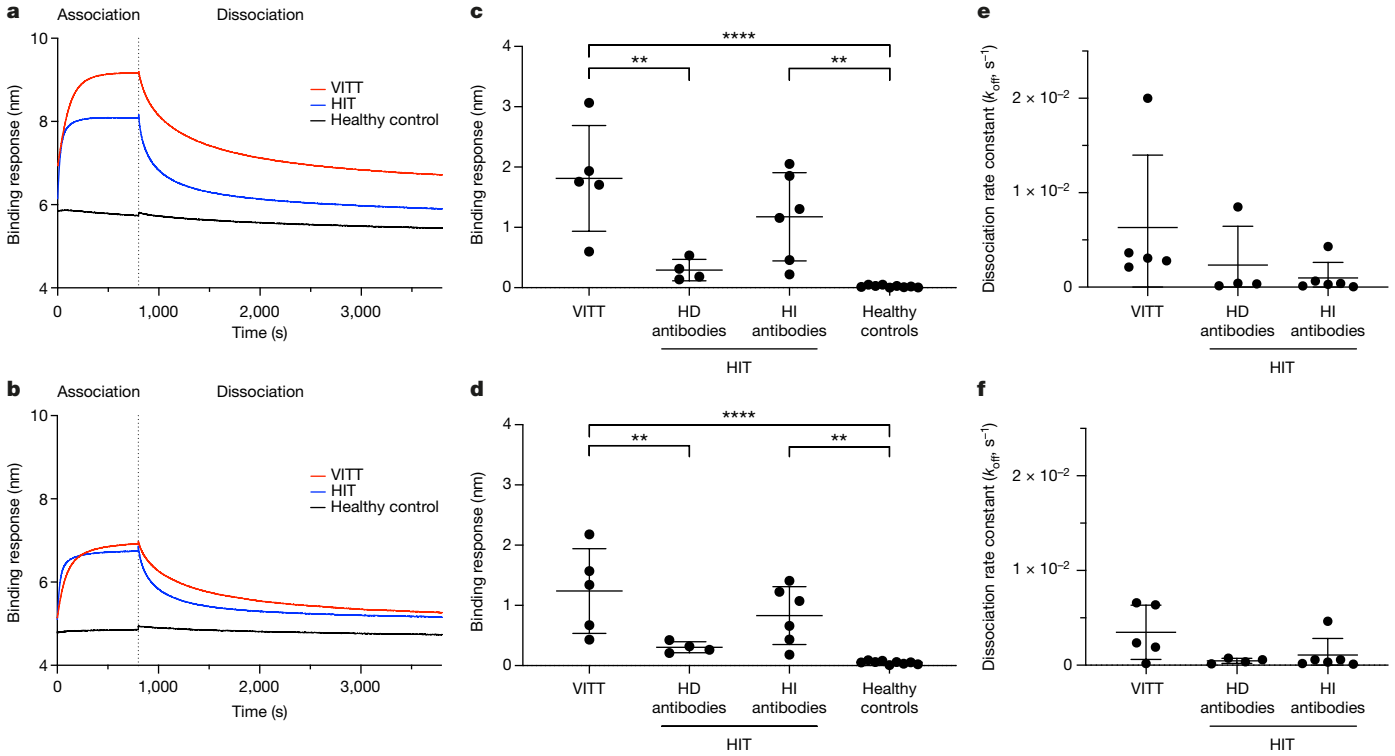

Fig. 2 | Association and dissociation of antibodies against PF4 and PF4–heparin using BLI. a, **b**, Representative binding responses of antibodies from a patient with VITT, a patient with HIT and a healthy control participant to biotinylated PF4 (**a**) and biotinylated PF4–heparin (**b**) immobilized on streptavidin biosensors. **c**–**f**, Binding responses with PF4 (**c**) and PF4–heparin (**d**) and dissociation rates (k_{off}) with PF4 (**e**) and PF4–heparin (**f**) of samples from patients with VITT ($n = 5$), patients with HIT with heparin-dependent antibodies ($n = 4$), patients with HIT with heparin-independent antibodies ($n = 6$) and healthy control individuals ($n = 10$). Patients with HIT with heparin-dependent antibodies ($n = 4$) are patients with typical HIT and patients with HIT with heparin-independent antibodies ($n = 6$) are patients with HIT who exhibited heparin-independent platelet activation in the SRA. The results show that VITT samples have higher binding responses to both PF4 and PF4–heparin than do HIT samples with heparin-dependent antibodies (PF4, $P = 0.0017$; PF4–heparin, $P = 0.0090$) and healthy control samples. Values are shown as the mean binding response (nm) ± s.d. and mean dissociation rate k_{off} (s^{-1}) ± s.d. HD antibodies, heparin-dependent antibodies; HI antibodies, heparin-independent antibodies. Statistical analysis by two-tailed Mann–Whitney test; $^{**}P < 0.01$, $^{****}P < 0.0005$.

with heparin-dependent antibodies, $P = 0.160$). The low dissociation rates probably allow for sufficient binding and formation of immune complexes to induce platelet activation by cross-linking FcγRIIa receptors on platelets. As human antibodies in serum are polyclonal, affinity could not be determined; however, binding responses and dissociation rates demonstrate the strength of the immune response and the avidity of the polyclonal samples, respectively.

Monoclonal antibodies against PF4, such as KKO (ref. [18]) and 1E12 (ref. [15]) facilitate the formation of ultra-large complexes of PF4 on the platelet surface. Previous studies using antibodies from patients with HIT[19] and monoclonal antibodies that resemble HIT antibodies[15] have shown that the higher affinity of heparin-independent antibodies in some patients with HIT can cluster PF4 tetramers and create platelet-activating immune complexes in the absence of heparin. Similarly, we found that anti-PF4 antibodies from patients with VITT have a comparable binding response and avidity to the antibodies from patients with HIT—especially patients with HIT with heparin-independent antibodies—implying that they can cluster PF4 tetramers and create the same ultra-large platelet-activating immune complexes (Extended Data Fig. 5).

It has been observed that 50% of HIT samples tested in the SRA cause platelet activation in vitro without the addition of heparin[20–22]. As such, the HIT samples were separated into two groups: those with heparin-dependent antibodies and those with both heparin-dependent and heparin-independent antibodies. The distinguishing feature that defines anti-PF4 antibodies from patients with VITT is that these patients—unlike patients with HIT—have not been exposed to heparin, and their anti-PF4 antibodies are restricted to the heparin-binding site.

In this report, we show that anti-PF4 antibodies in patients with VITT can induce platelet activation through FcγRIIa receptors in the presence of PF4, without heparin. However, other serum factors could also contribute to platelet activation. Previous studies found that antibodies from patients with VITT were able to activate platelets and cause platelet aggregation in the presence of adenoviral particles in a dose-dependent manner[1,23,24]. Thus, it is possible that platelet activation caused by anti-PF4 antibodies in patients with VITT is not the only factor that leads to the development of thrombotic events. HIT is also propagated by various pro-thrombotic mechanisms that could also be important in VITT, including Fc-receptor polymorphisms[25], monocyte activation and tissue factor production[26], and the generation of procoagulant microparticles[10].

This study offers an explanation for VITT-mediated platelet activation. The patients with VITT in our study exhibited similar antibody characteristics to one another and their antibodies bound PF4 at the same site as heparin. VITT antibodies form immune complexes without the addition of heparin or other co-factors, and activate platelets and potentially other cells through FcγRIIa receptors, which, in turn, could initiate coagulation at multiple points to cause thrombocytopaenia and thrombosis.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information,

acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03744-4.

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Methods

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Study participants

Participants included patients diagnosed with VITT ($n = 5$), patients diagnosed with HIT ($n = 10$) and healthy volunteers ($n = 10$). VITT diagnosis was based on four criteria: recent AstraZeneca vaccination; positive for anti-PF4 IgG antibodies; positive in the PF4-enhanced SRA; and no previous exposure to heparin. HIT diagnosis was confirmed based on three criteria: the 4Ts score, in which all patients with HIT had a clinical score of at least 4; a positive commercially available PF4-enhanced heparin-dependent IgG/A/M-specific EIA (Immucor, OD ≥ 0.45) and a positive SRA (SRA ≥ 20% ^{14}C-serotonin release)[27]. This study was approved by the Hamilton Integrated Research Ethics Board (HiREB) and informed written consent was obtained from all participants.

Platelet activation assays

Platelet activation assays were performed in the presence of heparin using the SRA, and including a modification in which increasing doses of exogenous PF4 were added, rather than heparin (PF4-SRA)[27,28]. Some assays were performed with high concentrations of unfractionated heparin (100 IU ml^{-1}), or with an Fc-receptor-blocking monoclonal antibody (IV.3).

Epitope mapping of antibody binding to PF4 from VITT and HIT samples using alanine-scanning mutagenesis

The full-length DNA coding sequence of human PF4 was cloned into the pET22b expression vector using restriction sites NdeI and HindIII (GenScript). The PF4 mutants were expressed and purified as previously described[5,29]. In brief, PF4 mutants were designed whereby non-alanine amino acids in wild-type PF4 were mutated to alanine and the alanine amino acids in wild-type PF4 were mutated to valine. PF4 mutants were introduced into *Escherichia coli* ArcticExpress (DE3) cells (Agilent Technologies). For the overexpression of PF4 mutants, cultures were grown at 37 °C to mid-exponential phase before induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 37 °C for 3 h. *E. coli* cells for each wild-type PF4 or PF4 mutant were lysed by sonication in 20 mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo Fisher Scientific), and 0.5% (w/v) sodium deoxycholate (MilliporeSigma) with 2 mM $MgCl_2$, 10 μg ml^{-1} DNaseI (MilliporeSigma) and EDTA-free protease inhibitor cocktail (Roche). The supernatant was then cleared by centrifugation at 40,000*g* for 40 min at 4 °C and applied onto a HiTrap Q HP column (Cytiva Life Sciences) equilibrated with 20 mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol and 5% (v/v) glycerol. The flow-through of the Q HP column was then stored at 4 °C, overnight. The following day, the serum was diluted twofold to yield a sodium chloride concentration of 200 mM with 20 mM sodium phosphate, pH 7.2, 1.4 mM β-mercaptoethanol and 5% (v/v) glycerol, syringe-filtered with a 0.2-μm filter (Acrodisc) and loaded onto a HiTrap Heparin HP column (Cytiva Life Sciences). Contaminants were eluted with 0.5 M sodium chloride and PF4 was eluted with a linear gradient from 0.5 to 2 M sodium chloride. Fractions containing pure wild-type or PF4 mutants were pooled, concentrated and buffer-exchanged to phosphate-buffered saline (PBS) and 1.5 M sodium chloride. The concentration of PF4 was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Protein expression and purity was assessed for each PF4 mutant using 4–18% denaturing SDS–PAGE.

The effect of the 70 amino acids on the binding of anti-PF4–heparin antibodies in patient samples was measured and analysed in a similar manner to that described previously[5]. The binding of anti-PF4–heparin antibodies to wild-type PF4 and PF4 mutants was measured using a modified PF4–heparin IgG-specific EIA[5,13]. Microtitre well plates (384 wells; Thermo Scientific Nunc) were coated with 10 μg ml^{-1} streptavidin and 1 IU ml^{-1} biotinylated-heparin and blocked with PBS supplemented with 3% (v/v) bovine serum albumin (BSA) for 2 h at ambient temperature. Wild-type PF4 or PF4 mutants at 5 μg ml^{-1} were then added and incubated for 1 h at ambient temperature. Diluted patient samples (1:50 prepared in 1% BSA in PBS) in technical duplicates were added to the plates and incubated for 1 h at room temperature. After washing, alkaline-phosphatase-conjugated goat anti-human IgG (γ-chain-specific, Jackson ImmunoResearch Laboratories) was added at a 1:3,000 dilution and incubated for 1 h at ambient temperature. The addition of 1 mg ml^{-1} para-nitrophenylphosphate (MilliporeSigma) substrate dissolved in 1 M diethanolamine buffer (pH 9.6) was used for detection. The OD was measured at 405 nm and 490 nm (as a reference) using a BioTek 800TS microplate reader (BioTek) to assess the binding of antibodies to wild-type PF4 and PF4 mutants. Results were reported as a percentage of the loss of binding relative to wild-type PF4 binding.

Heparin inhibition of VITT anti-PF4 antibodies

Microtitre well plates (96 wells, Nunc Maxisorp) were coated overnight at 4 °C with 100 μl per well of PF4 (60 μg ml^{-1}) diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6). The plates were then blocked with 200 μl per well of 3% (v/v) BSA prepared in PBS at room temperature for 2 h. VITT samples ($n = 4$) that were available were diluted 1:50 with 1% BSA in PBS and pre-incubated with increasing concentrations of unfractionated heparin (final concentrations of 0.1, 0.5, 1, 5 and 100 IU ml^{-1}; Pfizer) for 1 h at room temperature. The blocking solution was removed from the microtitre well plates and the VITT samples and heparin mixtures (100 μl per well) in technical duplicates were added to the plates, which were then incubated for 1 h at room temperature. The plates were washed twice with PBS–0.05% Tween 20 and three times with PBS. Bound human IgG antibodies were detected with 100 μl per well of alkaline-phosphatase-conjugated goat anti-human IgG (γ-chain-specific, 1:3,000, Jackson ImmunoResearch Laboratories) antibody prepared in 1% (v/v) BSA in PBS. Plates were washed as before and followed with the addition of 100 μl substrate (para-nitrophenylphosphate dissolved in diethanolamine buffer (MilliporeSigma)). The OD was measured at 405 nm and 490 nm (as a reference) using a BioTek 800TS microplate reader (BioTek).

Purification of total IgG antibodies

Total IgG antibodies from two VITT samples were purified for further analysis of binding kinetics. A volume of 2 ml protein G-coated sepharose beads (Thermo Fisher Scientific) was washed three times with PBS at room temperature, 300*g* for 5 min. Patient samples were heat-inactivated at 56 °C for 30 min and diluted three times in PBS. The samples were then transferred to protein G sepharose beads and incubated at room temperature for 1 h before extensive rinsing with 30 ml PBS. Total IgG was eluted from the protein G sepharose beads with 0.1 M glycine, pH 2.7 and neutralized by Tris buffer, pH 8.0.

Binding kinetics of VITT and HIT antibodies using BLI

Wild-type PF4 was labelled with biotin as previously described[30]. In brief, wild-type PF4 and PF4 mutants were incubated with 5× the volume of heparin sepharose 6 fast flow affinity chromatography medium (Cytiva Life Sciences) for 1 h with shaking at ambient temperature. EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) was added to the PF4 and heparin sepharose mixture in 20 molar excess and allowed to react for 1 h with shaking at ambient temperature. The biotinylated wild-type PF4 or PF4 mutants were eluted from the heparin sepharose using PBS and 2 M sodium chloride. Absorbance at 280 nm was measured using a spectrophotometer (Eppendorf AG) and used to calculate the concentration. Biotinylation of PF4 was checked using a streptavidin-coated anti-PF4–heparin EIA.

BLI experiments were performed using the Octet Red 96 instrument (FortéBio). Samples or buffer were dispensed into 96-well black flat-bottom microtitre plates (Greiner Bio-One) diluted in PBS supplemented with 1% (v/v) BSA at a volume of 200 µl per well with an operating temperature maintained at 23 °C. Streptavidin-coated biosensor tips (FortéBio) were hydrated with 1% BSA in PBS (MilliporeSigma) to establish a baseline before antigen immobilization for 60 s. Biotinylated recombinant PF4 (final concentration 7.5 µg ml^{-1}), alone or complexed with 0.125 IU ml^{-1} unfractionated heparin (LEO Pharma), was then immobilized on the biosensor tips for 1,200 s at 1,000 rpm followed by a baseline re-establishment for 1,800 s at 1,000 rpm. Antigen-coated sensors were then reacted with heat-inactivated patient samples or purified total IgG at a 1:32 dilution in duplicate for 780 s at 1,000 rpm followed by a dissociation step for 3,000 s at 1,000 rpm. Data were analysed using Octet User Software v.3.1 using the 2:1 heterogenous ligand-binding model. Reference values from control wells with buffer alone were subtracted and all results were aligned to the measured baseline. The binding profile response of each sample was expressed as the average wavelength shift in nm.

Data acquisition, statistical analysis and reproducibility

Differences between data were tested for statistical significance using the paired or unpaired *t*-test and the Mann–Whitney test. *P*-values are reported as two-tailed and $P < 0.05$ was considered to be statistically significant. All statistical analyses were conducted using GraphPad Prism v.9.1.0 (GraphPad Software). Experiments were repeated with technical duplicates independently twice with similar results unless otherwise stated.

Reporting summary

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

Data availability

The datasets generated during and/or analysed during the current study are not publicly available to allow for commercialization of research findings but are available from the corresponding author on reasonable request.

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Author contributions A.H. performed epitope mapping and BLI, analysed data and wrote the manuscript. J.G.K. and D.M.A. designed the research, interpreted data and wrote the manuscript. M.D. carried out the purification of PF4 mutants, performed epitope mapping and wrote the manuscript. I.N. designed the research, analysed and interpreted data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests A US provisional patent was submitted by I.N., D.M.A., J.G.K. and A.H. covering the products and methods for the diagnosis of VITT and the differentiation of VITT from HIT using the research in this study. The authors declare no other competing interests.

Additional information
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Correspondence and requests for materials should be addressed to I.N.
Peer review information *Nature* thanks Zaverio Ruggeri and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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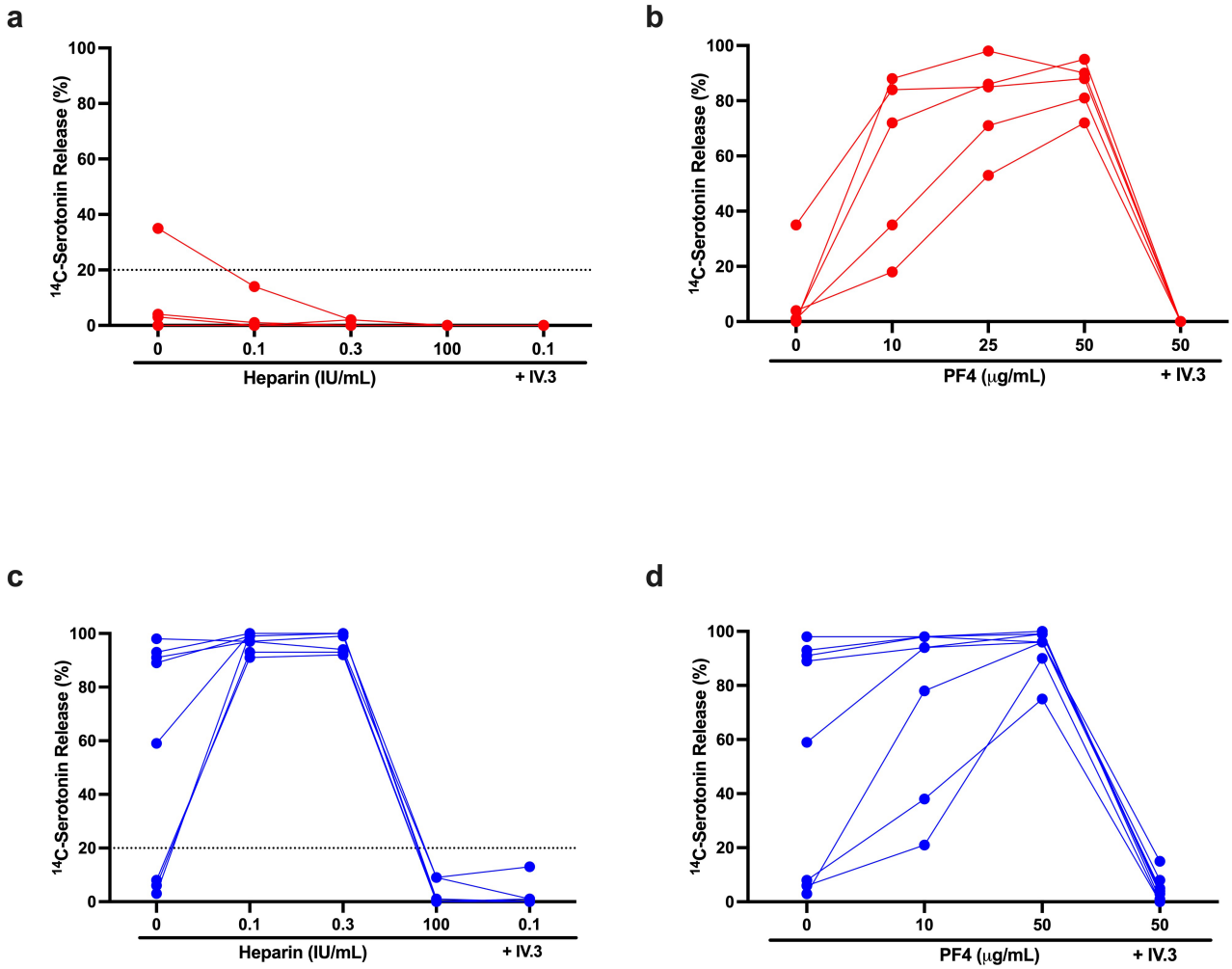
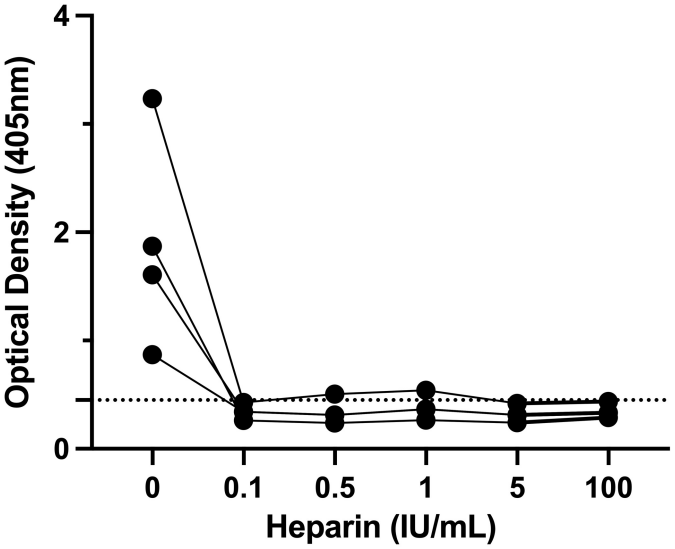
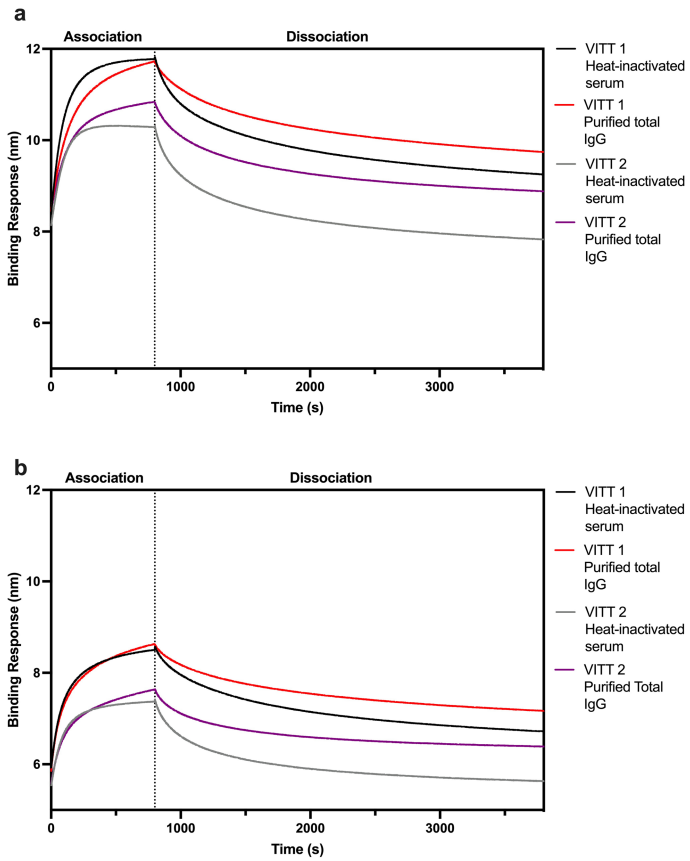


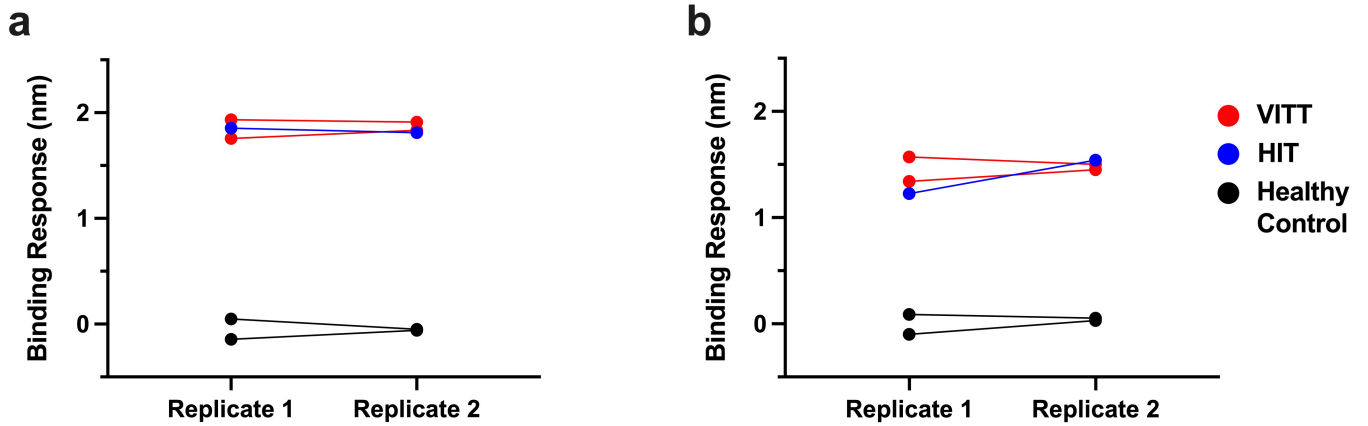
Extended Data Fig. 1 | Activation of platelets in samples from patients with VITT and patients with HIT in different SRAs. Platelet activation is shown as the percentage release of ^{14}C-serotonin. **a**, **c**, Samples from patients with VITT ($n = 5$; red lines) (**a**) and patients with HIT ($n = 10$; blue lines) (**c**) in the standard SRA[27]. **b**, **d**, The same VITT (**b**) and HIT (**d**) samples in the PF4-enhanced SRA[28,31] with added PF4. The results showed that in the standard SRA, the HIT samples activated platelets with ($n = 4$) or without ($n = 6$) heparin or with added PF4, whereas the VITT samples only activated platelets with the addition of PF4. Experiments were repeated with technical duplicates independently twice with similar results. All platelet activation was inhibited by the addition of the IV.3 monoclonal antibody that binds the FcγRIIa receptors on platelets.



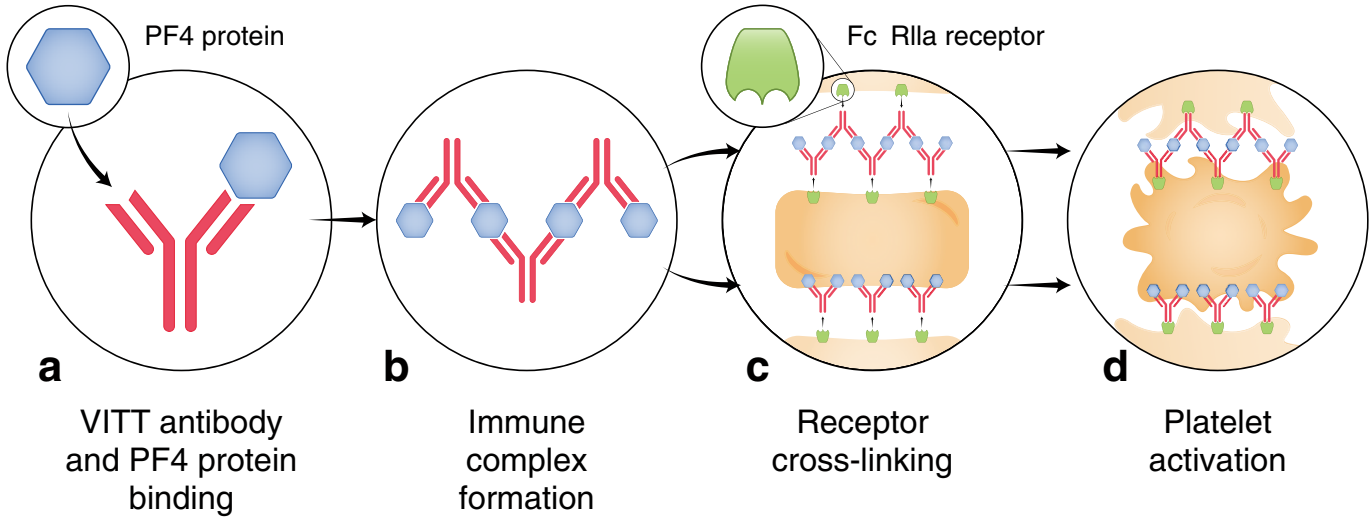
Extended Data Fig. 2 | Inhibition of VITT antibody binding to PF4 with increasing heparin concentrations. VITT samples ($n = 4$) were tested with increasing concentrations of heparin to investigate its effect on the binding of VITT antibodies to PF4. The experiment was repeated with technical duplicates independently twice with similar results. VITT antibody binding was disrupted by therapeutic concentrations and higher than therapeutic concentrations of heparin in all four VITT samples that were available for testing. Results are shown as the mean of the technical replicates. OD ≥ 0.45 is considered positive for anti-PF4 antibodies.



Extended Data Fig. 3 | BLI curves comparing binding kinetics in serum and purified total IgG of two VITT samples. Binding kinetics of VITT serum ($n = 2$) and its corresponding purified total IgG ($n = 2$) were compared to demonstrate anti-PF4 antibody specific responses. The experiment was repeated with technical duplicates independently twice with similar results. **a**, **b**, Association and dissociation steps are shown for biotinylated PF4 (**a**) and PF4–heparin (**b**) immobilized on streptavidin biosensor tips. Black lines represent the serum of VITT sample 1, red lines represent the purified total IgG of VITT sample 1, grey lines represent the serum of VITT sample 2 and purple lines represent the purified total IgG of VITT sample 2.



Extended Data Fig. 4 | Binding responses of replicate samples from patients with VITT, a patient with HIT and healthy control individuals. VITT ($n = 2$), HIT ($n = 1$) and healthy control ($n = 2$) samples were tested on different days to determine the reproducibility of the biolayer interferometry experiments. Experiments were repeated with technical duplicates independently three times with similar results. **a**, **b**, Binding responses against immobilized PF4 (**a**) and PF4–heparin (**b**) were measured as the average wavelength shift in nm. The binding response for each antigen and sample was reproducible on separate assays. Red points represent VITT samples, blue points represent the HIT sample and black points represent healthy control samples.



Extended Data Fig. 5 | Proposed mechanism for VITT antibodies binding to and clustering PF4 tetramers, independent of heparin, and forming platelet-activating immune complexes. a, **b**, We postulate that VITT antibodies bind the antigen, PF4 (**a**), which in turn can cluster PF4 tetramers and create platelet-activating immune complexes in the absence of heparin (**b**). **c**, **d**, The immune complexes can be found on the platelet surface and in solution, resulting in the aligning and close proximity of the Fc part of these antibodies, which then are able to cross-link FcγRIIa receptors (**c**) and lead to platelet activation (**d**).

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Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Biolayer interferometry (BLI) data was collected using the Octet® User Software version 3.1
Data analysis	All statistical analyses were conducted using GraphPad Prism (version 9.1.0, GraphPad Software)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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- Accession codes, unique identifiers, or web links for publicly available datasets
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- A description of any restrictions on data availability

The datasets generated during and/or analysed during the current study are not publicly available to allow for commercialization of research findings but are available from the corresponding author (Dr. Ishac Nazy) on reasonable request.

Field-specific reporting

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☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculations were performed. The sample sizes used in the study are sufficient as both vaccine-induced thrombotic thrombocytopenia and heparin-induced thrombocytopenia are rare disorders and it is difficult to acquire clinically defined patient samples. Sample size was determined based on the availability of positive VITT samples sent for testing at the McMaster Platelet Immunology Laboratory during the month of April.
Data exclusions	No datasets were excluded.
Replication	All assays were run with technical duplicates. Each assay had the same control sample run alongside all test subjects. Two heparin-induced thrombocytopenia samples and one vaccine-induced thrombotic thrombocytopenia were run in every assay on two separate occasions. Experiments were repeated with technical duplicates independently two times with similar results.
Randomization	Allocation was not randomized. Groups were defined by clinical diagnosis. Covariates were controlled by inspection of clinical data on each patient to ensure that there was no disorder overlap or any other diagnoses that could confound the data.
Blinding	Investigators were not blinded to group allocation during analysis. Blinding was not relevant to the study as all samples were run through every assay without discrimination.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Primary antibodies - VITT and HIT patient sample, healthy control samples (concentration 1/50 dilution) Secondary antibody - Alkaline-phosphatase conjugated goat anti-human IgG (γ-chain-specific, Jackson ImmunoResearch Laboratories, Inc, Westgrove, PA, USA), cat. no 109-056-098, lot no. 143072 (1/3,000 dilution)
Validation	VITT and HIT patient samples were selected from patients with confirmed diagnosis from the Platelet Immunology Laboratory. HIT patient samples were further confirmed clinically by an expert hematologist. Secondary antibody concentration used were based on previous optimizations by the lab found in the following reference: Horsewood, P. et al. Br J Haematol (1996). The secondary antibodies were validated by the manufacturer (see link https://www.jacksonimmuno.com/catalog/products/109-056-098). In summary, the antibody has been tested by ELISA and/or solid-phase absorbed to ensure minimal cross-reaction with bovine, horse, and mouse serum proteins.

Human research participants

Policy information about studies involving human research participants

Population characteristics	VITT patients (n = 5) had a mean age of 44 years (range: 35 – 72) and 2/5 were female. The time from first dose of the ChAdOx1 nCoV-19 vaccine to sample collection 14 to 40 days (mean 28 days). HIT patients (n=10) had a mean age of 69 years

	(range: 52 – 81 years) and 5/10 were female. Nine of 10 HIT patients (90%) experienced thrombosis. The time from heparin initiation to sera collection was 6 – 27 days (mean 14.3 days).
Recruitment	Sera from patients with vaccine-induced thrombotic thrombocytopenia and heparin-induced thrombocytopenia were referred to the McMaster Platelet Immunology Laboratory for confirmatory diagnosis. We tested consecutive samples as they came in. It is possible that there is self-selection bias as the samples sent for testing may be extreme cases of VITT in terms of clinical features.
Ethics oversight	Hamilton Integrated Research Ethics Board (HiREB)

Note that full information on the approval of the study protocol must also be provided in the manuscript.