Platelet-Neutrophil Aggregate formation induces NLRP3 inflammasome activation in vaccine induced thrombotic thrombocytopenia (VITT)

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HYPOTHESIS

Formation of platelet-leukocyte aggregates (PLAs) and inflammasome activation are common features of thromboinflammatory diseases. Here we hypothesized that PLA formation and inflammasome activation might be involved in the development of VITT.

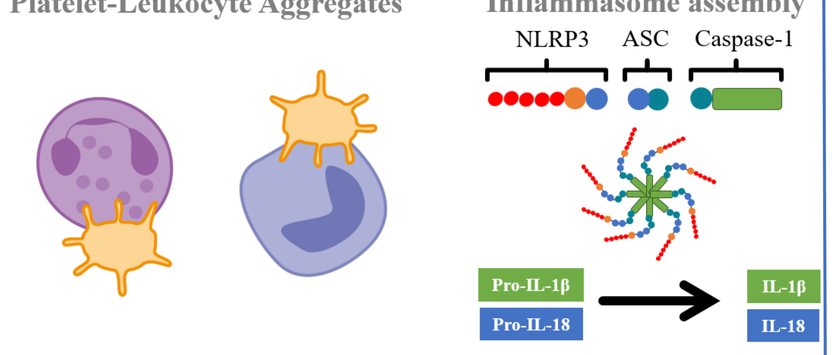


Figure 1 – Hypothesis.

STUDY DESIGN

Samples of Individuals (n=57) with post-vaccine thrombosis (PVT) were collected in the entire Brazilian territory as part of pharmacovigilance. Samples from individuals vaccinated with adenovirus vector vaccines (AVV) were sorted according guidelines by the likelihood that these events are VITT related (NEJM. 385:1680-1689, 2021). Age/sex matched unvaccinated (UV) individuals were also included (CAAE-48532621.8.0000.5262/52396621.0.0000.5262).

Criteria:

- Presence of thrombosis
- Onset of symptoms 5-30 days after vaccination
- Thrombocytopenia (<150.000 platelets/μL)
- Positive Anti-PF4 antibodies (ELISA)
- D-dimer >4000μg/mL (>2000μg/mL)

D-dimer <2000μg/mL = exclusion criteria
Unknown D-dimer = possible

Definite VITT:
- All criteria met

Probable VITT:
- 4 criteria
- 3 criteria with D-dimer 2000 - 4000μg/mL

Possible VITT:
- 3 criteria
- 2 criteria with D-dimer 2000 - 4000μg/mL

Unlikely VITT:
- 2 criteria

Figure 2 – VITT classification. Defined by the occurence of thrombosis between 5-30 days post vaccination associated with thrombocytopenia, high D-dimer and presence of anti-PF4 Ab.

METHODOLOGY

COHORT

57 individuals that presented with thrombosis following AVV (PVT) were sorted according to the number of VITT criteria met. 20 were classified as unlikely VITT, 7 as possible VITT, 18 as probable VITT and 12 as definite VITT. The samples were compared with samples from 28 age and sex matching unvaccinated individuals (**Figure 3**).

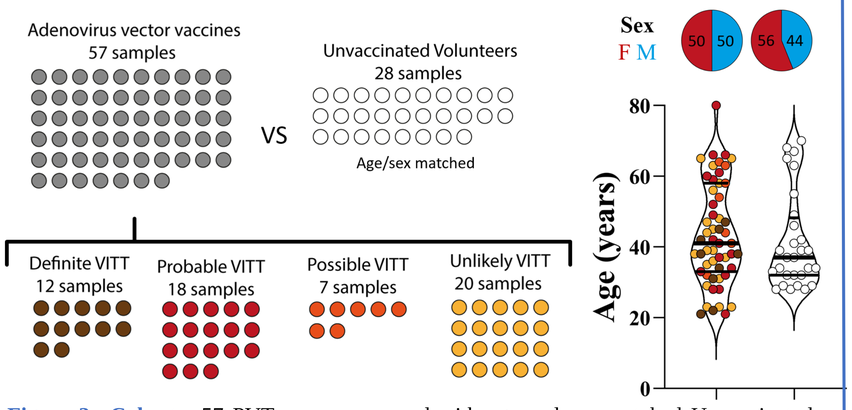
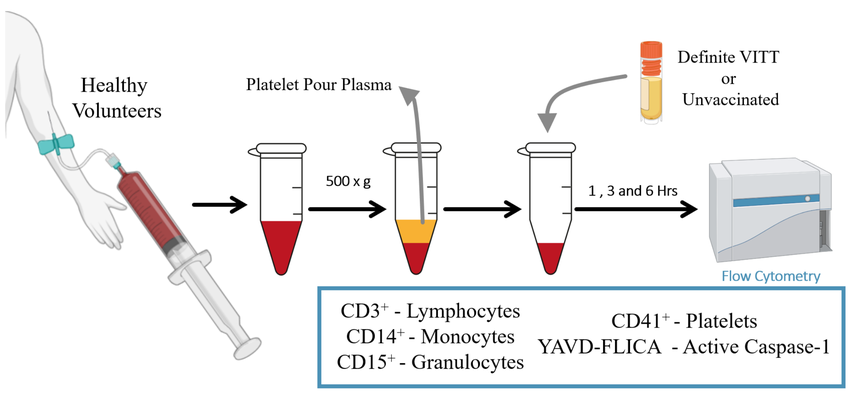


Figure 3– Cohort. 57 PVTs were compared with age and sex matched Unvaccinated Volunters. Median, 25th and 75th quartiles and range are shown.

Ex-vivo experiment



To investigate PLA assays, we performed ex-vivo plasma exchange substituting the platelet pour plasma (PPP) from healthy volunteers' (HV) whole blood with PPP from DV or Unvaccinated individuals for 1, 3 and 6 hours, and evaluating through flow cytometry the PLA formation and inflammasome activation.

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RESULTS

Thromboinflammation in PVTs

PVT patients as a whole had lower platelet counts, elevated D-dimer levels and lower fibrinogen levels (**Figure 4D-F**).

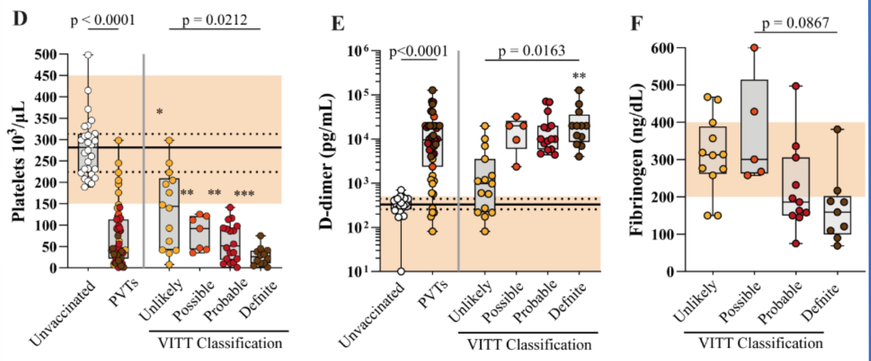


Figure 4 – Hypercoagulability of patients with postvaccination thrombosis (PVT). Platelet count (D) and plasma concentrations of (E) D-dimer, (F) fibrinogen.

As expected, PVTs also displayed higher plasmatic levels of the thromboinflammatory molecules p-selectin (CD62p) and Tissue Factor **(Figure 4G and H).**

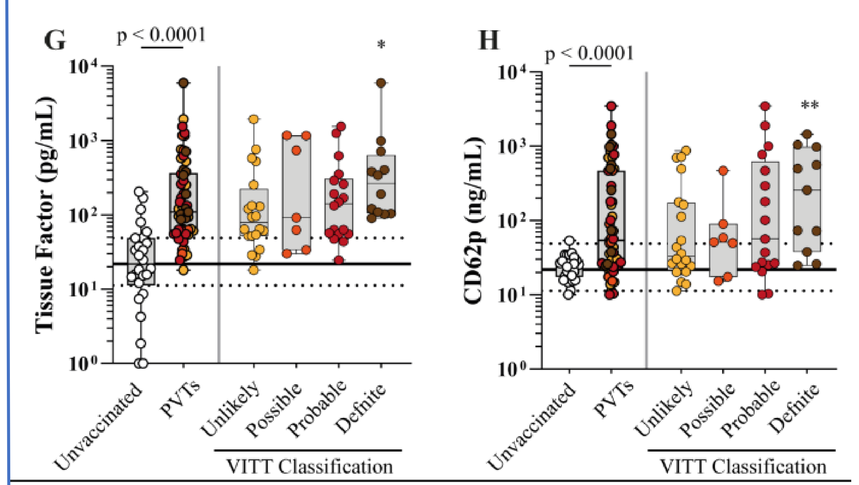


Figure 4 (continued)– Hypercoagulability of patients with postvaccination thrombosis (PVT). (G) tissue factor and (H) CD62p of Unvaccinated (n=28) and PVTs (n=57) according to their VITT classification. The normal clinical range is displayed in the orange area, boxes represent the median and 25th and 75th quartiles, and whiskers display the range. Asterisks indicate significant differences in patients with different VITT classifications compared to the unvaccinated group. *p<0.05, **p<0.01 and ***p<0.001. The black solid and ticked lines represent the median and 25th and 75th quartiles, respectively, of the unvaccinated group.

Inflamassome activation in PVTs

Inflamassome assembly, followed by Caspase-1 activation and secretion of IL-1β and L-18 are characteristics of VITT.

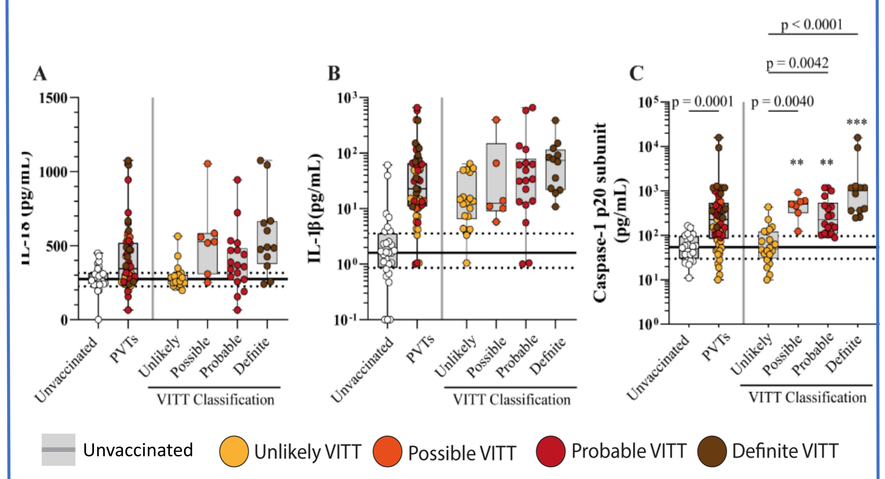


Figure 5 –Inflamassome Activation in Definite VITT. Definite VITT cases presented higher and IL-18 (A) and IL-1β (B) then Unlikely and Caspase-1 (C) in the plasma of PVTs stratified according to VITT likelihood classification. Median, 25th and 75th quartiles and range are shown. Grey areas and lines represent de interquartile range and median of the unvaccinated controls.

The VITT milieu induces platelet-granulocyte aggregate formation and caspase-1 activation

At all-time points, Definite VITT plasma induced increased activation of caspase-1 in granulocytes (CD15+) and platelet-granulocyte aggregate (PGA) formation was increased at 3 and 6 hours after incubation with VITT plasma.

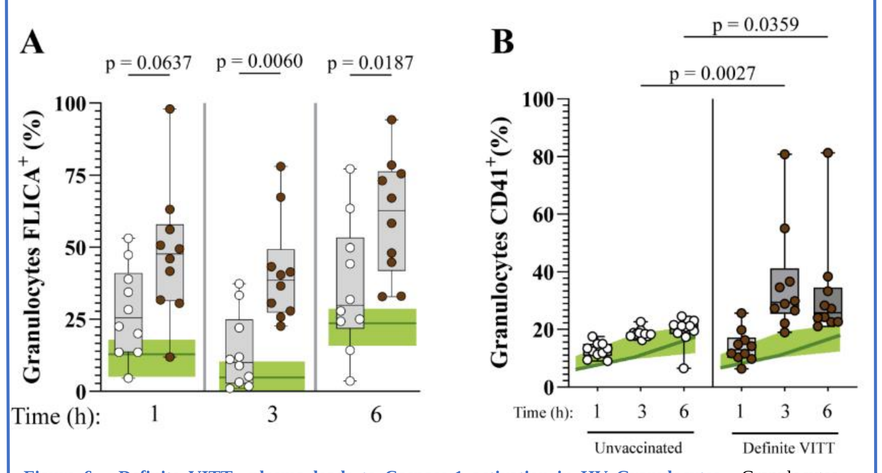
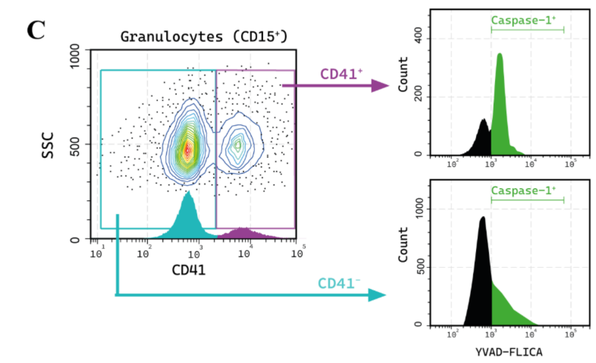


Figure 6 – Definite VITT plasma leads to Caspase-1 activation in HV Granulocytes. Granulocytes isolated from healthy donnors and incubated with VITT plasma were analysed by flow cytometry. Percentage of HV CD15+ cells expressing the active form of Caspase-1 after incubation with Definite VITT plasma for 1, 3 or 6 hours. Median, 25th and 75th quartiles and range are shown.



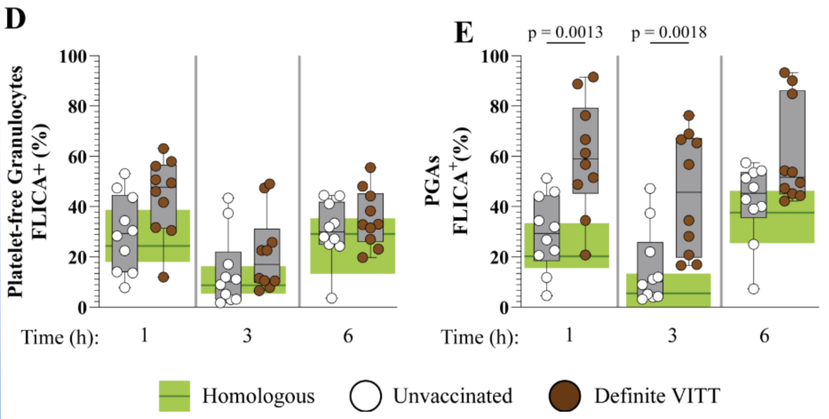


Figure 6 (continued) - (C) The gating strategy was used to determine the expression of caspase-1 in platelet-free granulocytes (CD15+/CD41-) or PNAs (CD15+/CD41+). In platelet-free granulocytes, caspase-1 activation was not increased in the blood reconstituted with Definite VITT plasma compared to the plasma from Unvaccinated individuals (**D**), but platelet-granulocyte aggregates had increased activation of caspase-1 (**E**) after incubation with 50% of Definite VITT plasma.

PNA formation triggers caspase-1 activation in cells exposed to VITT plasma. PNA formation occurs through FcγRIIa and caspase-1 activation through the NLRP3 inflammasome

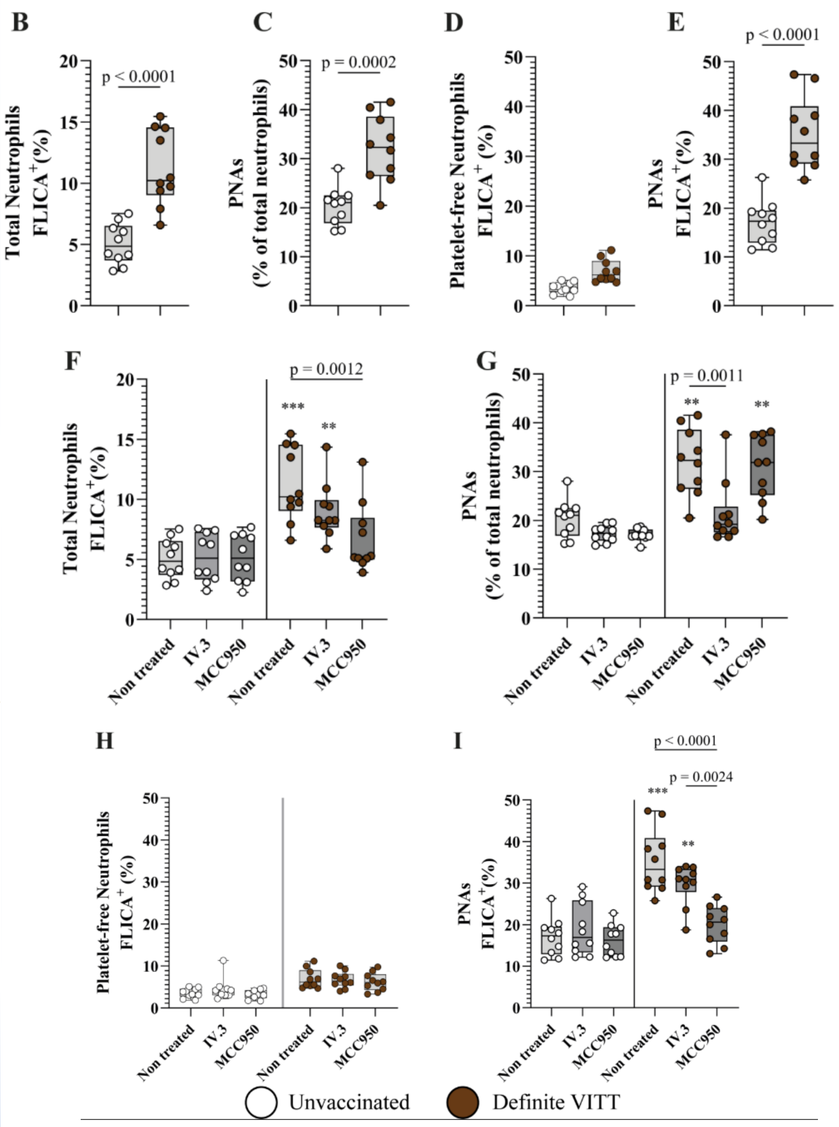


Figure 7– Definite VITT plasma induces aggregation through FCγRIIa- and NLRP3 inflammasome-dependent caspase-1 activation. Isolated platelet-neutrophils were stimulated with 50% of Definite VITT or unvacinnated individuals plasma. (B) Percentage of total neutrophils aggregated with platelets. (C) Percentage of platelet-free neutrophils and (D) PNAs expressing the active form of caspase-1 after incubation with 50% plasma from unvaccinated or definite VITT patients for 3 hours. We used an FcγRII neutralizing antibody (IV.3) or an NLRP3 inhibitor (MCC950) prior to incubation with VITT or unvacinnated plasma. (F) Percentage of total neutrophils expressing active caspase-1, (G) percentage of neutrophils aggregated with platelets, (H) percentage of platelet-free neutrophils and (I) percentage of PNAs expressing caspase-1 after 30 minutes of pretreatment with IV.3 or MCC950 (platelets and neutrophils) followed by incubation with 50% plasma from Definite VITT (n=10) or unvaccinated (n=10) individuals.

CONCLUSION

Individuals that developed VITT after immunization with adenovirus vector based SARS-CoV-2 vaccines displayed elevated IL-1β, IL-18 and activated Caspase-1 in the plasma, indicating that inflamassome assembly is a characteristic of VITT. Our data also suggest that factors present in the plasma of VITT patients are capable of promoting the formation of platelet-neutrophil aggregates in a FcγRIIa-dependent manner, which in turn leads to the activation of pro-caspase-1 via the NLRP3 inflammasome.

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