The contribution of platelet glycoprotein receptors to inflammatory bleeding prevention is stimulus and organ dependent

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Supplemental Information

Methods

Mice

GPVI$^{-/-}$[1, 2], *Clec1b*$^{fl/fl}$PF4cre[3], double KO (DKO), Pdpn$^{fl/fl}$VAV1cre^{+4}, hIL4R/GPIbα-tg[5], VWF$^{-/-}$[6] have been described previously. The lung inflammation model was approved by the Animal Care and Use Committee of the Claude Bernard Institute (Paris); the rpA model was undertaken under UK Home Office licence 70/8359 and the Animal Care and Use Committee of the Claude Bernard Institute.

Lung inflammation model

Sedated mice were inoculated intranasally with 20 µg LPS from *Pseudomonas aeruginosa* prepared in 60 µL of saline (30 µL/nostril). Bronchoalveolar lavage (BAL) was performed 24 hours later by canulating the trachea with an 18-gauge angiocath and lavaging the lungs 2 times with 1 mL sterile PBS supplemented with 1% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA). The BAL fluid was then centrifuged (8 min at 2000*g*) and pelleted cells were resuspended in 75 µL PBS. A fraction of the resuspended cells was used for analysis in flow cytometry after combined fluorescent staining of CD45 and Ly6G for determination of neutrophil counts. The remaining fraction of cells was lysed by sonication to release haemoglobin from red blood cells, centrifuged (5 min at 10,000*g*), and haemoglobin concentration measured in supernatants as described[7].

For induction of the rpA reaction in lungs, mice were inoculated intranasally with 60 µg rabbit anti-BSA IgG prepared in 60 µL of saline (30 µL/nostril) immediately followed by IV injection of BSA (50 µg/g mouse) in 50 µL saline.

Cutaneous reverse passive Arthus reaction

The cutaneous rpA was induced in the back skin of mice as described previously[7]. Briefly, the reaction was elicited by intradermal injection of rabbit anti-BSA IgG (60 µg in 20 µL saline, 2 spots/mouse) immediately followed by intravenous injection of BSA (50 µg/g mouse) in saline. Mice were

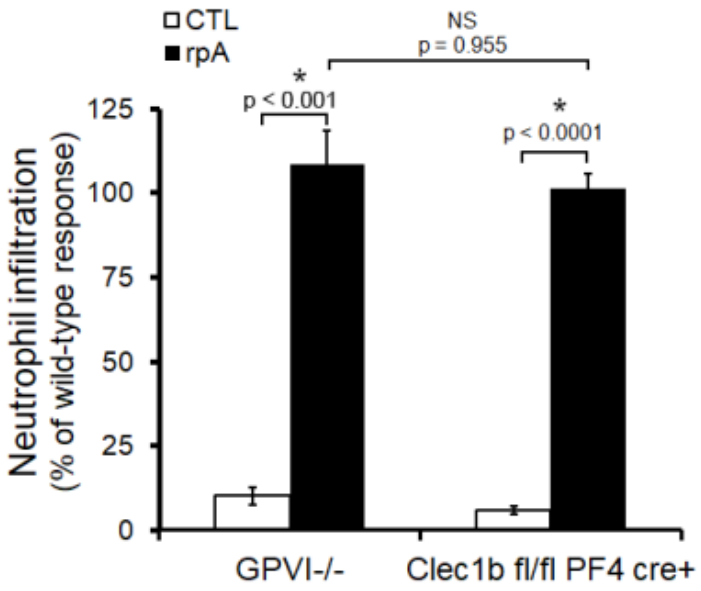
euthanized after 4 hours and skin biopsies at control and reaction sites harvested for haemoglobin determination and/or myeloperoxyidase content[7].

Preparation of functionalized fluorescent microspheres

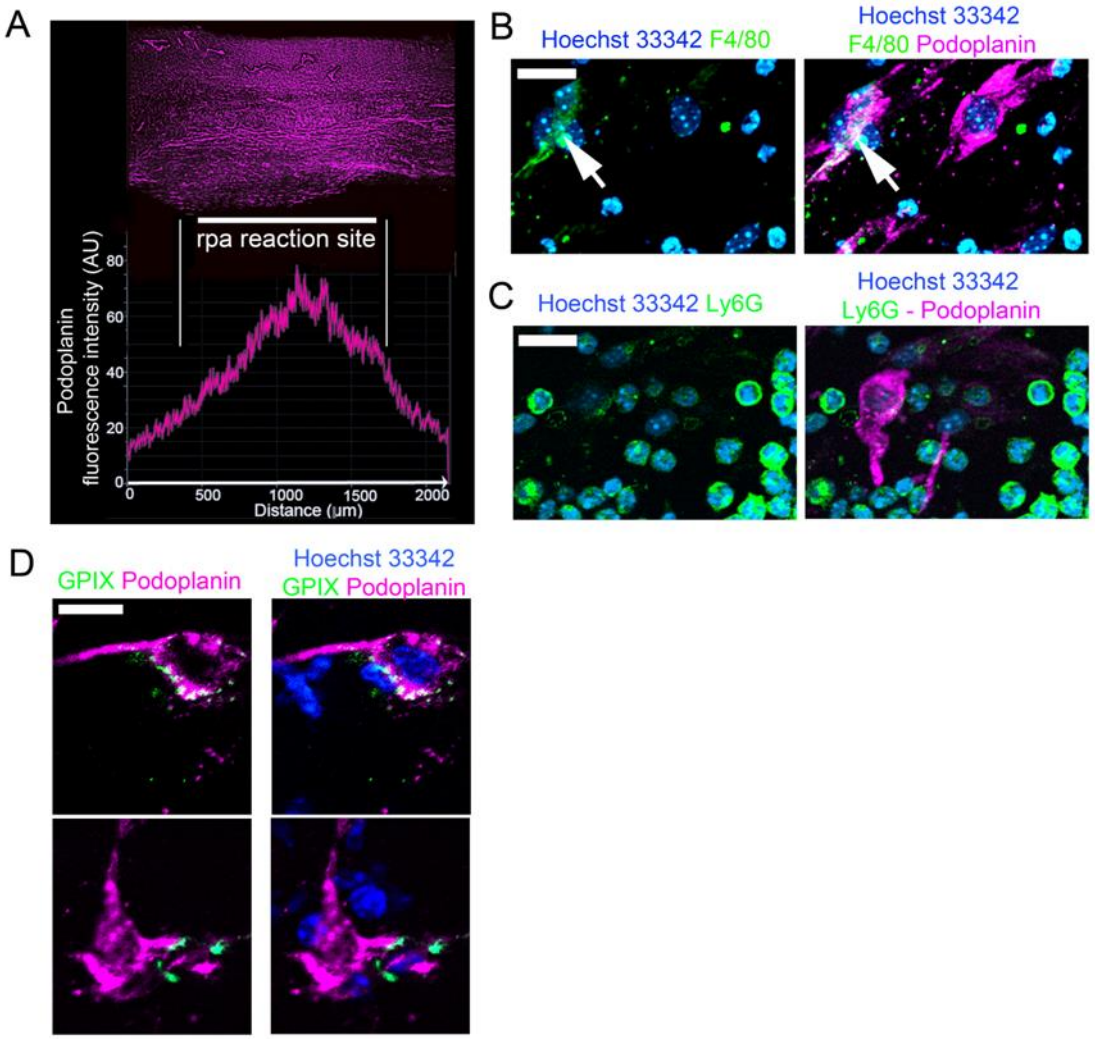
Yellow Green, Nile Red, Crimson, or Blue fluorescent microspheres (FluoSpheres Carboxylate-Modified Microspheres, 1 µm-diameter, Molecular Probes, Life Technologies, Saint Aubin, France) were coated at 2 µg of IgG/cm^2 bead surface area following the manufacturer's instructions. The following antibodies were used for coating: rabbit anti- mouse collagen IV (PA1-26148, Thermo Fisher Scientific), hamster anti-mouse podoplanin (mAb 8.1.1), and sheep anti-mouse vWF (Abcam 11713). Microspheres were injected at 200.10^6 beads/mouse in saline.

Statistics Data are expressed as mean ± standard error of the mean and were compared by the Mann-Whitney *U* test. *P* values <0.05 were regarded as statistically significant.

Supplemental Figures



Supplemental Figure 1. Comparison of neutrophil recruitment to the skin during the cutaneous rpA between GPVI$^{-/-}$ and platelet CLEC2-deficient mice. Quantification and comparison of neutrophil recruitment in control and rpA challenged skin biopsies as assessed by measurement of skin myeloperoxidase (MPO) content. Results are expressed as percent relative to the mean MPO content measured in rpA-challenged skin biopsies of control GPVI+/+ wild-type mice. n = 5-9 mice per group.



Supplemental Figure 2. Podoplanin expression in the skin during the cutaneous rpA reaction. A. Representative image of a full thickness immunofluorescence staining for podoplanin (purple) in WT inflamed skin. The corresponding fluorescence intensity profile measured along a line-scan drawn parallel to the skin surface is shown below the image. **B.** Immunofluorescence staining for nuclei (blue, Hoechst 33342), macrophages (green, F4/80) and podoplanin (purple) in the inflamed skin of a WT mouse. White arrow shows a podoplanin-expressing macrophage. Bar = 20 µm. **C.** Immunofluorescence staining for nuclei (blue, Hoechst 33342), neutrophils (green, Ly6G) and podoplanin (purple) in the inflamed skin of a WT mouse. Bar = 20 µm. **D.** Immunofluorescence staining for nuclei (blue, Hoechst 33342), platelets (green, GPIX) and podoplanin (purple) in the inflamed skin of GPVI$^{-/-}$ mice. Bar = 10 µm.

References

1. Kato K, Kanaji T, Russell S, et al. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. Blood. 2003;102:1701-1707.
2. Bender M, Hagedorn I, Nieswandt B. Genetic and antibody-induced glycoprotein VI deficiency equally protects mice from mechanically and FeCl(3) -induced thrombosis. J Thromb Haemost. 2011;9:1423-1426.
3. Finney BA, Schweighoffer E, Navarro-Nunez L, et al. CLEC-2 and Syk in the megakaryocytic/platelet lineage are essential for development. Blood. 2012;119:1747-1756.
4. Hitchcock JR, Cook CN, Bobat S, et al. Inflammation drives thrombosis after Salmonella infection via CLEC-2 on platelets. J Clin Invest. 2015;125:4429-4446.
5. Kanaji T, Russell S, Ware J. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. Blood. 2002;100:2102-2107.
6 Hillgruber C, Steingraber AK, Poppelmann B, et al. Blocking von Willebrand factor for treatment of cutaneous inflammation. J Invest Dermatol. 2014;134:77-86.
7. Gros A, Syvannarath V, Lamrani L, et al. Single platelets seal neutrophil-induced vascular breaches via GPVI during immune-complex-mediated inflammation in mice. Blood. 2015;126:1017-1026.