# A subset of anti-HLA antibodies induces FcγRIIa-dependent platelet activation

**Maaike Rijkers,[1] Anno Saris,[2] Sebastiaan Heidt,[3] Arend Mulder,[3] Leendert Porcelijn,[4] Frans H.J. Claas,[3] Ruben Bierings,[1] Frank W.G. Leebeek,[5] A.J. Gerard Jansen,[1,5] Gestur Vidarsson,[6] Jan Voorberg[1,7] and Masja de Haas[3,4,8]**

[1]Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [2]Department of Immunopathology, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [3]Department of Immunohaematology and Blood Transfusion, Leiden University Medical Center; [4]Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam; [5]Department of Hematology, Erasmus University Medical Center, Rotterdam; [6]Department of Experimental Immunohematology, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [7]Department of Vascular Medicine, Amsterdam UMC, University of Amsterdam and [8]Center for Clinical Transfusion Research, Sanquin, Leiden, the Netherlands

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Correspondence: m.rijkers@sanquin.nl

# Supplemental Data

# Supplemental Materials

**Materials**

Antibodies directed against CD62P (clone AK-4), CD41/CD61 (clone PAC-1), HLA A/B/C (clone W6/32), human IgG Fc (clone HP60717) and appropriate isotype controls were obtained from BioLegend (San Diego, CA, USA). Mouse anti-SPARC (sc-73472) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-Human IgG F(ab')2 Secondary Antibody was from Thermo Fisher Scientific (Waltham MA USA). Blocking antibody Anti-CD32a (clone IV.3) was from Bio X Cell (West Lebanon, NH, USA). Syk Inhibitor IV (BAY 61-3606), Syk inhibitor I and Syk inhibitor II were obtained from Merck (Kenilworth, NJ, USA). Nanogam Intravenous immunoglobulin (IVIg) was from Sanquin (Amsterdam, The Netherlands). Calcein Violet-AM was from BioLegend and Calcein Green-AM from Thermo Fisher Scientific. PKH26 membrane dye was from Sigma (Zwijndrecht, The Netherlands). PAR1-activating peptide (PAR1-AP) SFLLRN-$NH_2$ was from Peptides International (Louisville, KY, USA). Pierce™ F(ab')$_2$ Preparation Kit was from Thermo Fisher Scientific.

**HLA monoclonal antibodies and patient sera**

Human HLA-specific monoclonal antibodies (HLA mAbs), all of IgG1 isotype, were produced by hybridoma technology as described previously.[1,2] Antibodies were purified by protein A chromatography. F(ab')2 fragments of HLA mAbs were generated using a F(ab')2 Preparation Kit (PierceTM Thermo Fisher).

Blood samples of patients refractory to platelet transfusion were sent to the Department of Immunohematology Diagnostic Services, Sanquin, Amsterdam, the Netherlands. Leftover material was used according to the Dutch established codes of conduct for responsible use of patient material and as approved by our institute. HLA antibody specificities in patient sera were determined by single antigen bead assay (Luminex). Thirteen sera positive for HLA antibodies and negative for other platelet specific antibodies were used.

**Alpha-granule release analysis (SPARC Western blot and VWF ELISA)**

After incubation with HLA mAbs, platelets were spun down and supernatant was collected. Sample buffer (62.5mM Tris-HCl, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, pH 6.8) and 10 mM DTT was added to the supernatant. Proteins were separated on SDS PAGE gels (NuPAGE® Novex® 4-12% Bis-Tris, Thermo Scientific) and proteins were blotted on nitrocellulose membranes using the iBlot® 7-Minute Blotting System (Thermo Scientific). Odyssey® Blocking Buffer (PBS) (LI-COR Biotechnology, Lincoln, NE, USA) was used to block the membranes, following incubation with mouse anti-SPARC and secondary antibody IRDye® 680LT Donkey anti-Mouse IgG (H+L) (LI-COR Biotechnology).

Concentrations of von Willebrand factor (VWF) were determined in the supernatants which were collected from platelets incubated with HLA mAbs. A VWF ELISA was performed as described previously.[3] In short, a mix of 4 primary murine antibodies directed to VWF (RAgMix, composed of

CLB-RAg 20, CLB-RAg 35, CLB-RAg42 and CLB RAg 56) were used to coat a MaxiSorp microtiter plate in coating buffer (50 mM NaHCO3, pH 9.8) for 16h at 4°C. Supernatant, diluted in PBS containing 0.01% Tween20 and 1% bovine serum albumin, was incubate for 2h at 37°C. As secondary antibody polyclonal peroxidase-labeled anti-VWF IgG (DAKO A/S, Glustrup, Denmark) was used. Normal human plasma from a pool of 30 donors was used as standard.

**Platelet agglutination/aggregation**

Maximum percentage of aggregation/agglutination of washed platelets (250 µl of $2.5x10^8$ platelets/ml in Tyrode's buffer) was determined by employing light transmission aggregometry on an aggregometer (Chrono-log, model 700). HLA mAbs were used at increasing concentrations (2.5-20 µg/ml) and aggregation was inhibited by pre-incubation of the platelets with IV.3 or Syk inhibitor IV. For priming experiments a combination of 4 µg/ml HLA mAbs and 0.05 µg/ml PAR1 AP or 0.5 µg/ml collagen (Chronolog Corp, Havertown, PA, USA).

**Inter-/intra-platelet activation assays**

Donor platelets with an HLA typing either matched or not-matched with the specificities of HLA mAb WIM8E5 were selected and labeled with calcein-AM green or calcein Violet-AM. Calcein-labeled platelets ($2.5*10^8$ platelets/ml) of 2 donors (differently labeled) were mixed 1:1 and incubated with HLA mAbs or PAR1 activating peptide for 1h at RT. Platelets were fixed with 1% PFA. Interaction between platelets with different labels was measured directly by measuring events positive for calcein-green and calcein-violet employing flow cytometry. In addition, platelets were stained with CD62P antibody and analyzed employing flow cytometry.

**Platelet opsonization and internalization by macrophages**

Citrated blood from HLA typed healthy individuals was collected and platelet rich plasma (PRP) was obtained by centrifugation at 200 *g* for 10 min. PRP was washed and labeled 20 min in PBS at RT with 3.75 *µ*M PKH26 membrane dye (Sigma, Zwijndrecht, the Netherlands) with continuous gentle shaking. Subsequently, platelets were incubated for 60 min with 10 *µ*g/ml WIM8E5, SN607D8, or SN230G6, in the presence or absence of 5 *µ*M SYK inhibitor IV and subsequently washed with sequestrine buffer (17.5 mM $Na_2HPO_4$, 8.9 mM $Na_2EDTA$, 154 mM NaCl, pH 6.9, containing 0.1% [wt/vol] bovine serum albumin, all obtained from Merck Millipore, Amsterdam, the Netherlands). Internalization of platelets by macrophages was determined as previously described.[4] In short, monocytes isolated from fresh apheresis using the Elutra Cell Separation System (Gambro, Lakewood, CO, USA) were differentiated into macrophages with 10 ng/ml granulocyte–macrophage CSF (Cellgenix, Freiburg, Germany) in IMDM culture medium (Lonza, Breda, the Netherlands) supplemented with 10% fetal calf serum (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, and 100 U/ml streptomycin (both from Gibco/Thermo Fischer Scientific, the Netherlands) at 37°C and 5% $CO_2$. At day 8, macrophages were incubated at 37˚C with PKH-labeled platelets. After 60 min, macrophages were harvested using 130 mM lidocaine (Sigma) with 10 mM EDTA (Merck Millipore), washed twice in PBS, fixed with 3.7% paraformaldehyde (Sigma) in PBS, washed in PBS+0.5%BSA, and incubated 30 min at RT with FITC-labeled anti-CD61 (Beckman Coulter, Woerden, the Netherlands) and APC-

labeled anti-HLA-DR (both from BD biosciences, Breda, the Netherlands). Subsequently, cells were analyzed using imaging flow cytometry (ImageStream®X Mark II Imaging Flow Cytometer, Merck Millipore) and IDEAS Application (IDEAS software V6.1.303.0, Merck Millipore). Gating strategy (See Supplementary Figure S6) involved selection of single macrophages (aspect ratio intensity vs. area; for bright-field and APC) in focus (gradient RMS; for bright field and APC channel) and exclusion of false positive cells (defined as intracellular fluorescence of the platelet-specific anti-CD61 membrane staining). Next, to determine platelet internalization qualitatively, PKH+ macrophages were selected and an intracellular mask (based on HLA-DR membrane staining) was used to establish whether the PKH fluorescence was intracellular or not. Platelet internalization by macrophages was quantified by measuring the amount of PKH fluorescence within macrophages (using fluorescence intensity within an intracellular mask based on HLA-DR membrane staining).

## Supplemental Figure S1

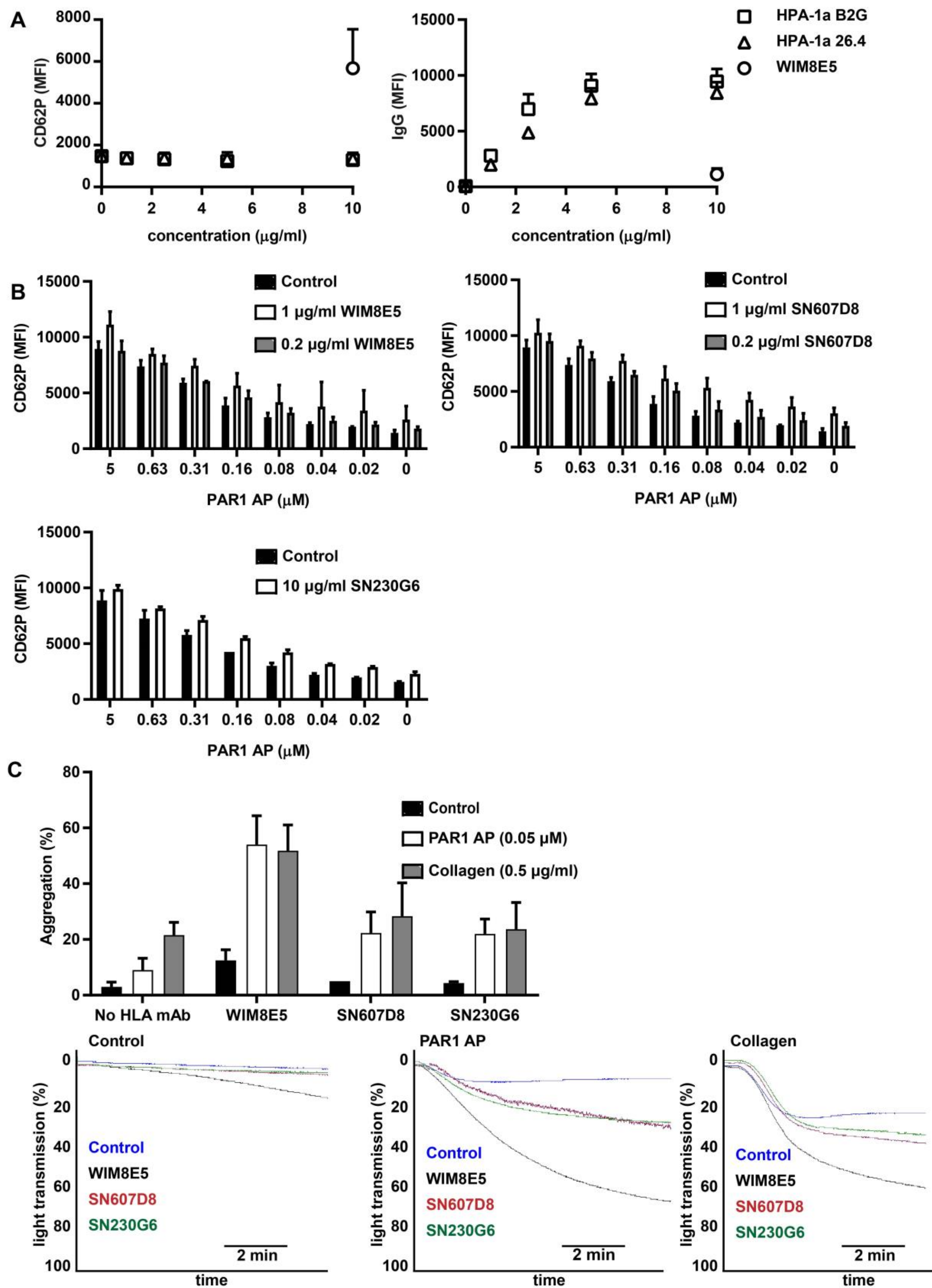


**Figure S1: Anti-HPA1a antibodies and priming effect of HLA mAbs on agonist induced platelet activation.** (A) Platelets were incubated with 1, 2.5, 5 and 10 µg/ml anti-HPA-1a antibodies (clone B2G[5,6] (produced as described by Dekkers et al.[7]) and clone 26.4[8]). Binding of the antibodies was

evaluated using anti human IgG antibody, and platelet activating properties of HPA-1a mAbs were assessed using an anti-CD62P antibody. As control platelets were incubated with 10 μg/ml WIM8E5. (B) Platelets were incubated with increasing concentration of PAR1-AP in combination with not-activating HLA mAb (SN607D8 10 μg/ml) and low concentrations of activating HLA mAbs (1 or 0.2 μg/ml SN607D8, 1 or 0.2 μg/ml WIM8E5). No synergistic effect on CD62 exposure was observed following simultaneous stimulation of platelets with PAR1-AP and HLA mAbs WIM8E5 and SN607D8. (C) Platelet aggregation was performed with suboptimal concentrations of PAR1-AP (0.05 μM) and collagen (0.5 μg/ml) in presence and absence of 4 μg/ml WIM8E5, SN607D8 and SN230G6. A combination of PAR1-AP and HLA mAbs WIM8E5, SN607D8 and SN230G6 induced enhanced platelet aggregation, which was most pronounced for WIM8E5. Similar results were obtained for collagen. Representative aggregation curves are shown.

## Supplemental Figure S2

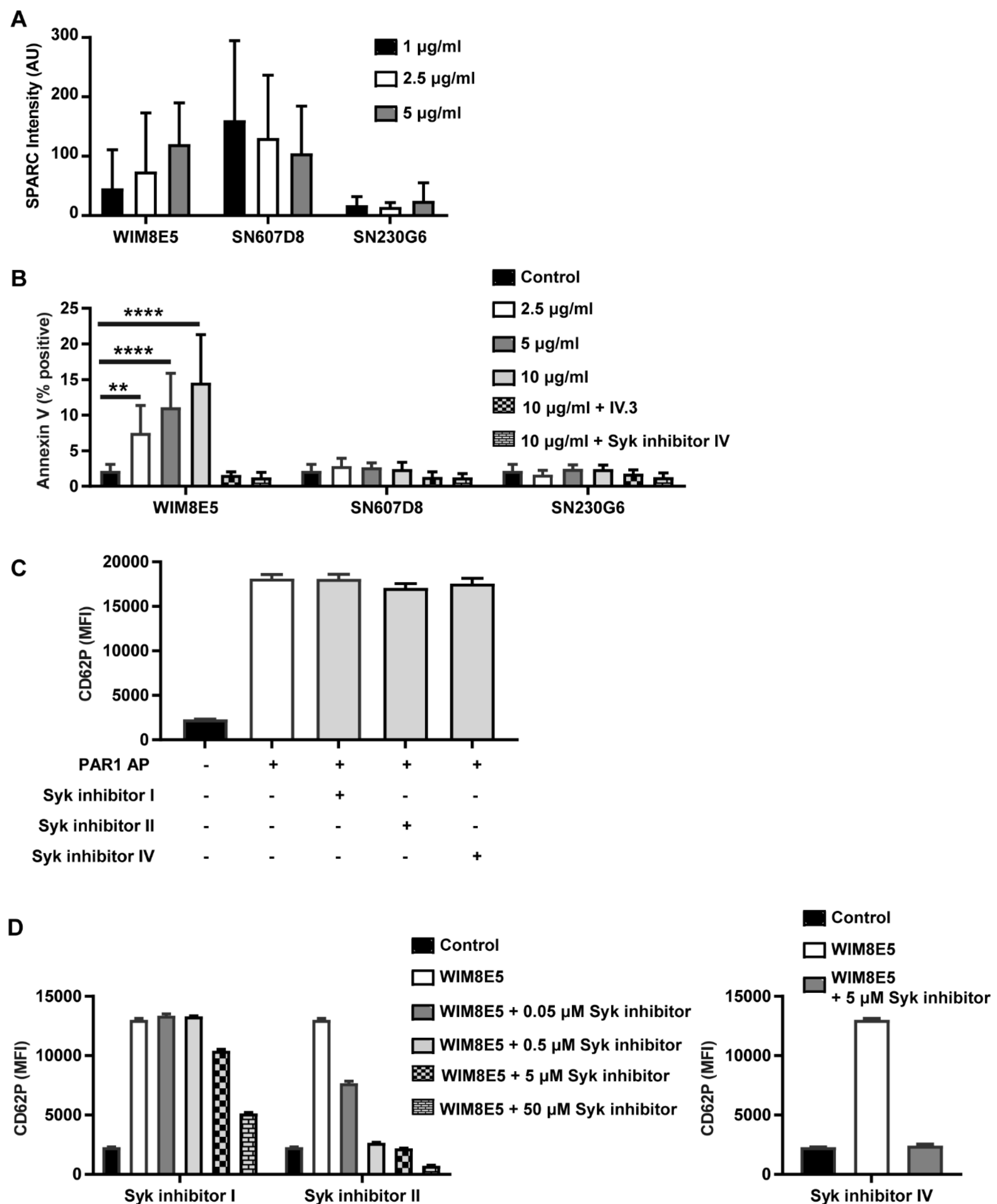


**Figure S2: Further characterization of HLA mAbs induced platelet activation.** (A) Densitometric quantification of HLA mAbs induced SPARC release. Densitometry was performed on SPARC Western blot using Image Studio Lite Software (Version 4.0). Intensities were normalized to control (buffer only, no HLA mAbs added). (B) Phosphatidylserine (PS) exposure induced by HLA mAbs. Platelets were incubated with 2.5, 5, 10 µg/ml WIM8E5, SN607D8 or SN230G6. To inhibit FcγRIIa-dependent signaling, platelets were pre-incubated with 10 µg/ml IV.3 or 5 µM Syk inhibitor IV. Phosphatidylserine

(PS) exposure was measured employing annexin V. Annexin V was incubated with HLA mAbs treated platelets in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Exposed PS was assessed by FACS analysis. WIM8E5 induced FcγRIIa-dependent PS exposure (as revealed by annexin V binding), SN607D8 and SN230G6 did not induce significantly enhanced PS exposure (C) Specificity of Syk-inhibitors. To test specificity of Syk inhibitors, platelets were pre-incubated with 5 µM Syk inhibitor I, II or IV, and then PAR1-AP was added for 1h at RT. CD62P exposure was measured as platelet activation marker. At a concentration of 5 µM, all 3 Syk inhibitors had no effect on PAR1-AP induced α-granule release, suggesting specific inhibition of Syk-dependent pathways. (D) Platelets were pre-incubated with Syk inhibitor I, II or IV, following 10 µg/ml WIM8E5 for 1h at RT. Platelet activation was measured by CD62P surface exposure. Similar to Syk inhibitor IV, also Syk inhibitor I and II inhibit WIM8E5-induced CD62P exposure.

# Supplemental Figure S3

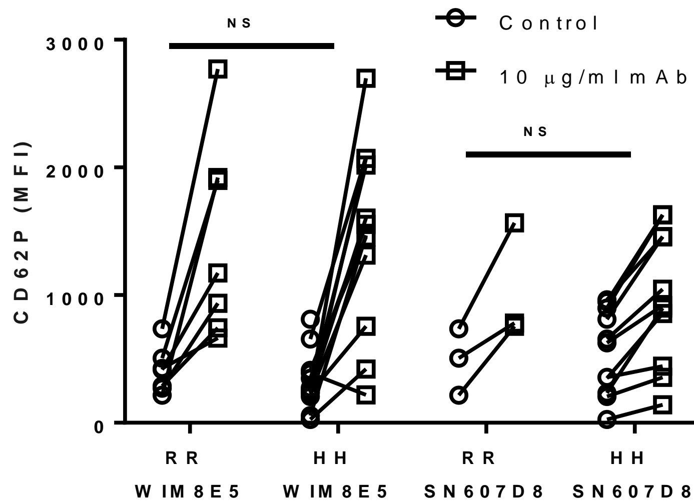


**Figure S3. Impact of the R131H SNP of FcγRIIa on HLA mAbs induced platelet activation.** Rollin et al. proposed a role for a polymorphism at position 131 in FcγRIIa in the pathogenesis of HIT.[9] Either a histidine (H) or arginine (R) is present at position 131 in FcγRIIa; the R131H polymorphism has been shown to affect the affinity of IgG2 for FcγRIIa. The affinity of IgG2 for FcγRIIa is four fold higher for the 131H variant ($4.5 \pm 2 * 10^5$ $M^{-1}$) when compared to the 131R variant ($1 \pm 0.1 * 10^5$ $M^{-1}$).[10,11] We compared the ability of HLA mAbs to induce activation of platelets from donors homozygous for the 131H or the 131R variant of FcγRIIa. No significant differences were found in CD62P exposure induced by WIM8E5 and SN607D8 between platelets from donors expressing FcγRIIa-131H or 131R. These results indicate the FcγRIIa 131SNP does not affect platelet activation by HLA antibodies of IgG1 subclass such as WIM8E5 and SN607D8. Each line represents a different platelet donor. Paired ANOVA with Tukey's multiple comparison test. *: $P<0.05$, **: $P<0.01$.

## Supplemental Figure S4

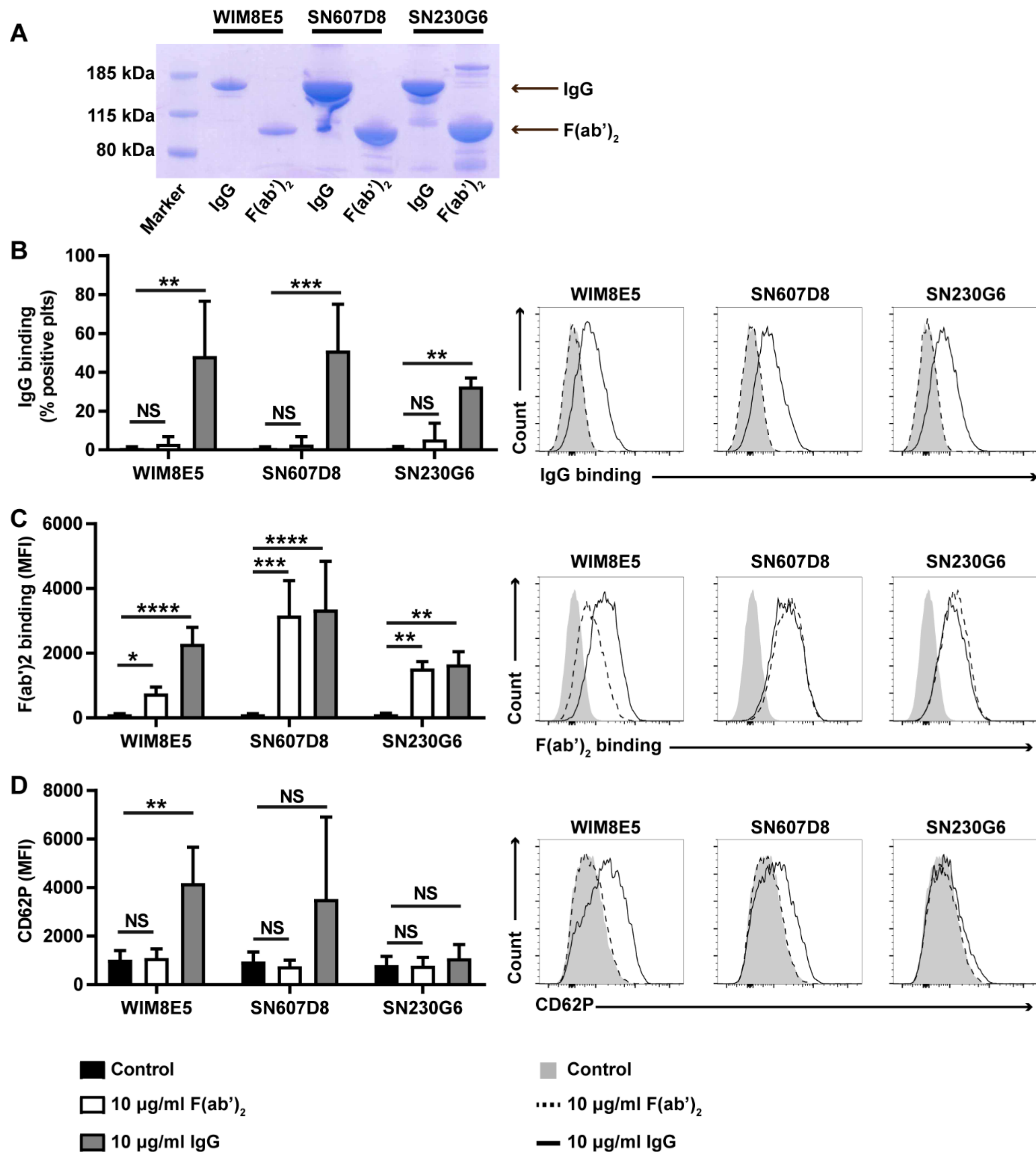


**Figure S4: F(ab')2 fragments of HLA mAbs do not induce FcγRIIa-dependent activation.** (A) WIM8E5, SN607D8 and SN230G6 IgG and corresponding F(ab')2 fragments were separated on a SDS page gel (NuPAGE® Novex® 4-12% Bis-Tris, Thermo Scientific) and stained with Imperial Blue stain. Intact IgG has an apparent molecular weight of 150 kDa and F(ab')2 fragments of 110 kDa. (B): Binding of F(ab')2 fragments and IgGs to platelets as revealed by a secondary anti-Fc antibody. As expected in this set-up binding is only observed upon incubation of platelets with undigested IgG. (C): Binding of F(ab')2 fragments and IgGs to platelets as revealed by a secondary anti-F(ab')2 antibody. As expected in this set-up binding is observed upon incubation of platelets with undigested IgG as well as F(ab')2 fragments. (D): CD62P surface exposure upon incubation of platelets with undigested IgG and F(ab')2

fragments. Platelet activation as measured by CD62P exposure is only observed for WIM8E5 IgG and SN607D8 IgG. No CD62P exposure is observed for the non-activating SN230G6 antibody. F(ab')2 fragments of WIM8E5, SN607D8 and SN230G6 do not induce CD62P exposure. All samples were compared to control (buffer only, no HLA antibodies). Data are given as mean ± SD. Paired ANOVA with Tukey's multiple comparison test. *: $P<0.05$, **: $P<0.01$, ***: $P<0.005$, ****: $P<0.001$. Representative FCM plots are shown

## Supplemental Figure S5

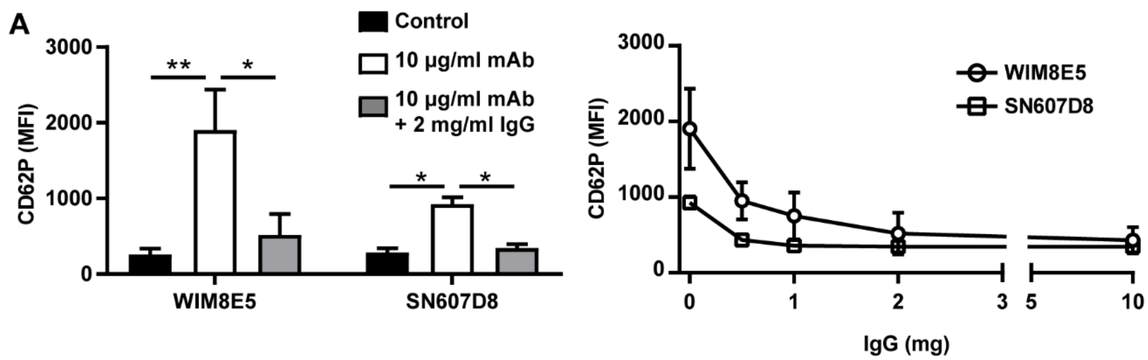


**Figure S5: IVIg inhibits HLA mAb induced CD62P exposure** (A): Platelets were pre-incubated with or without IVIg before addition of WIM8E5 or SN607D8. CD62P surface exposure was measured employing FCM and compared to control (buffer only). WIM8E5 and SN607D8 induced CD62P exposure could be block completely by the addition of 2 mg/ml IgG. In the right panel a dose response curve is shown for both WIM8E5 and SN607D8. Mean ± SD. Paired ANOVA with Tukey's multiple comparison test. *: $P<0.05$, **: $P<0.01$.

# Supplemental Figure S6

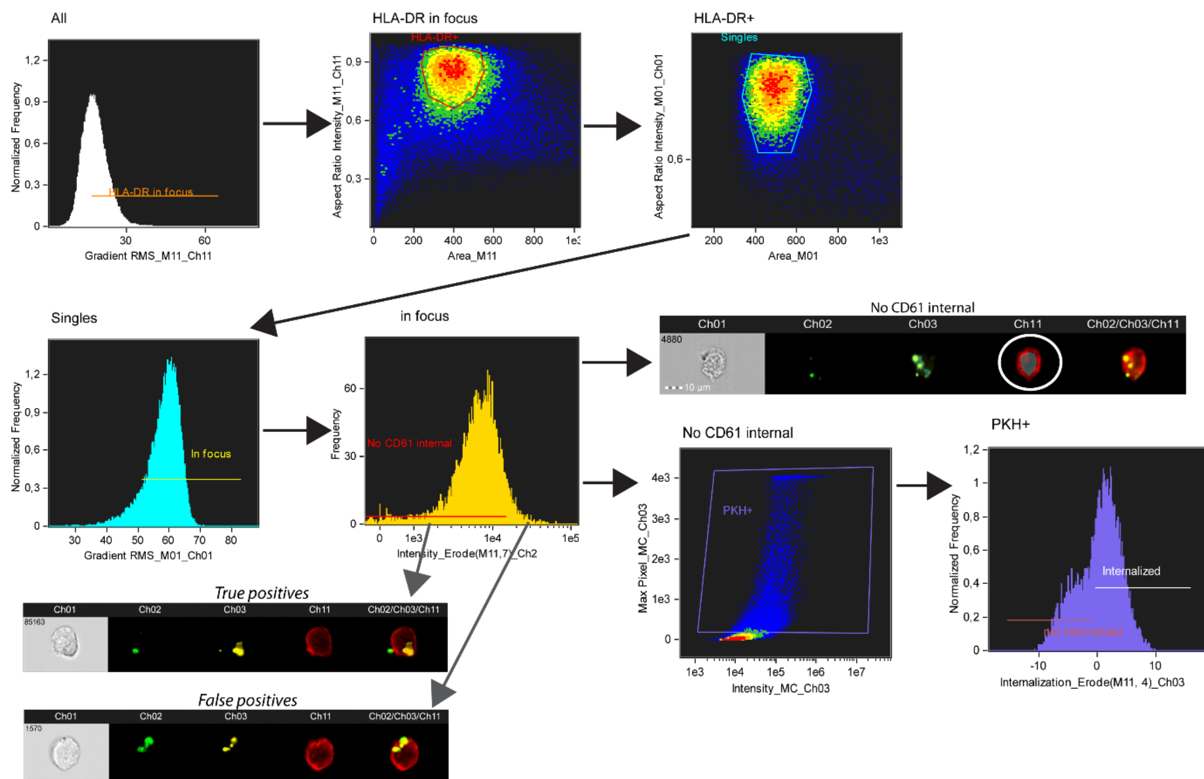


**Figure S6: Imaging flow cytometry gating strategy used to determine platelet internalization by macrophages.** First, the single cells in focus were selected using HLA-DR staining and subsequently using bright field. Platelets were labeled using the dye PKH. False positive cells were excluded using CD61 staining (Ch02). Because the CD61 staining was performed after incubation with platelets, only surface bound platelets can be stained and intracellular CD61 staining is therefore considered false positive (due to focal plane limitations). Typical true and false positive examples are depicted. Platelet internalization was quantified by determining the intracellular fluorescence by selecting PKH$^+$ macrophages using the depicted mask (within white circle). Ch01 = bright field, Ch02 = CD61 staining, Ch03 = PLT PKH staining, Ch11 = HLA-DR staining.

## Supplemental Figure S7

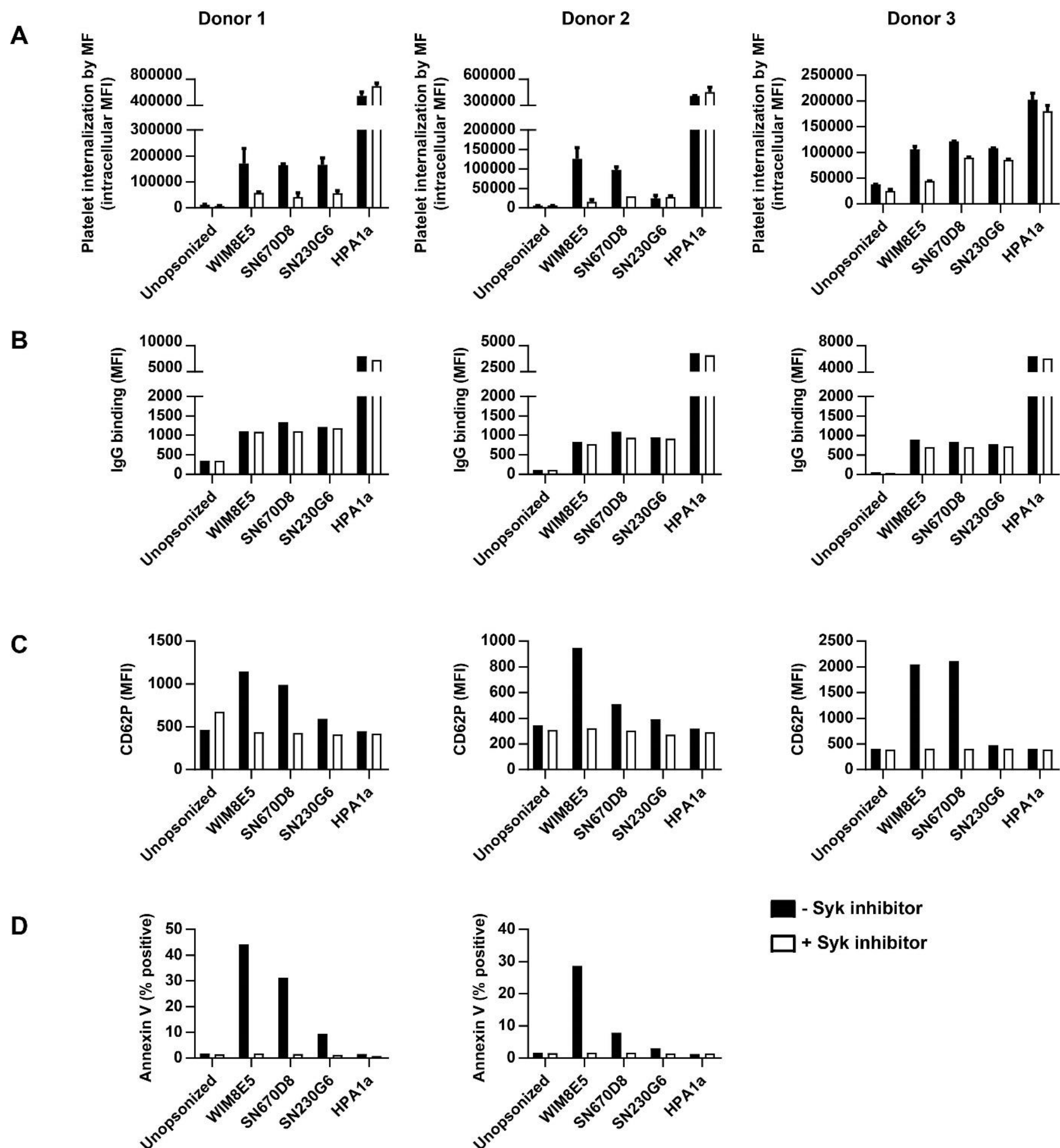


**Figure S7: Phagocytosis by macrophages of platelets opsonized by HLA or anti-HPA1a antibodies.** Data are shown for 3 separate experiments using platelets of 3 different donors. Platelets were incubated with 10 µg/ml WIM8E5, SN607D8 or SN230G6 in the presence or absence of 5 µM Syk inhibitor IV. Opsonized platelets were incubated for 1h with monocyte-derived macrophages and internalization was analyzed employing imaging flow cytometry. (A) Platelet uptake of fluorescent macrophages expressed as intracellular platelet (PKH) fluorescence per macrophage. Opsonization of platelets by WIM8E5, SN670D8 and SN230G6 promotes uptake by macrophages. Syk-dependent enhancement of platelet uptake was observed for WIM8E5, SN607D8 and SN230G6 for platelets of donor 1. For platelets derived of donor 2 Syk-dependent enhancement of phagocytosis was observed

upon treatment of platelets with WIM8E5 and SN670D8. For this donor no enhanced uptake of platelets was observed upon incubation of platelets with SN230G6. For donor 3 Syk-dependent enhancement of phagocytosis was observed for WIM8E5. No clear Syk-dependence was observed for phagocytosis of platelets incubated with SN670D8 and SN230G6. For this particular donor phagocytosis of HLA mAbs opsonized platelets appeared to be only partially dependent on Syk. As a control we also monitored the uptake of the anti-HPA1A opsonized platelets by macrophages. Anti-HPA-1a opsonized platelets were readily phagocytosed. Phagocytosis of anti-HPA-1a opsonized platelets by macrophages was independent of Syk. (B) Control for IgG binding to platelets. After opsonization of platelets with antibodies, samples of platelets were collected to measure IgG binding to the platelets. The results show that WIM8E5, SN670D8, SN230G6 and HPA-1a bind to platelets of donor 1, 2 and 3. As expected binding is independent of pre-incubation of platelets with Syk inhibitor IV. (C) CD62P exposure following incubation of antibodies with platelets. Increased CD62P exposure is observed for platelets of donor 1 and 2 upon incubation with WIM8E5, SN670D8 and SN230G6. For donor 3 increased CD62P exposure is observed for WIM8E5 and SN670D8. HPA-1A did not induce CD62P exposure on platelets of donor 1,2 or 3. (D) Phosphatidylserine (PS) exposure (as measured employing annexin V binding). PS exposure was observed upon incubation of platelets of donor 1 and 2 with WIM8E5 and SN670D8. Low levels of PS were exposed upon incubation of platelets of donor 1 and 2 with SN230G6. No PS exposure was observed following incubation of platelets of donor 1 and 2 with HPA-1a. PS exposure was not assessed for the experiments performed with platelets of donor 3.

# Supplemental Table 1

| Serum nr | Antibody specificities | Donor 1 HLA A1 A2 B51 Matching antibodies: | Donor 2 HLA A1 A2 B35 B62 Matching antibodies: |
|---|---|---|---|
| Serum 1 | A2 A23 A24 A25 A26 A29 A31 A32 A33 A34 A43 A66 A68 A69 A74 B7 B13 B27 B35 B37 B42 B47 B48 B49 B50 B53 B54 B55 B56 B57 B58 B60 B61 B62 B63 B72 B67 B73 B81 B82 | A2 | A2 B62 B35 |
| Serum 2 | A2 A23 A24 A25 A32 B13 B27 B35 B37 B38 B39 B44 B47 B49 B51 B52 B53 B54 B56 B57 B58 B59 B63 B67 B71 B75 B77 B78 | A2 B51 | A2 B35 |
| Serum 3 | A1 A2 A11 A23 A24 A25 A26 A29 A30 A31 A32 A33 A34 A36 A43 A66 A68 A69 A80 B8 B13 B18 B27 B35 B37 B38 B39 B44 B45 B47 B49 B51 B52 B53 B54 B57 B58 B59 B63 B64 B65 B71 B76 B77 B78 B82 | A1 A2 B51 | A1 A2 B35 |
| Serum 4 | A1 A3 A11 A23 A24 A25 A26 A30 A31 A32 A34 A36 A43 A66 A74 A80 B7 B8 B18 B27 B35 B37 B38 B42 B46 B49 B50 B51 B52 B53 B54 B55 B56 B57 B58 B59 B62 B63 B67 B71 B72 B73 B75 B76 B77 B81 B82 | A1 B51 | A1 B62 B35 |
| Serum 5 | A1 A11 A23 A24 A25 A26 A29 A30 A31 A32 A33 A34 A36 A43 A66 A68 A69 A74 A80 B8 B18 B27 B35 B37 B38 B39 B41 B42 B44 B47 B49 B51 B52 B53 B54 B55 B57 B58 B59 B63 B64 B65 B67 B73 B77 B82 | A1 B51 | A1 B35 |
| Serum 6 | A1 A2 A3 B76 B44 B45 B57 B82 | A1 A2 | A1 A2 |
| Serum 7 | A1 A2 A3 A11 A23 A24 A29 A30 A31 A32 A36 A43 A69 A74 A80 B7 B13 B27 B42 B44 B45 B47 B48 B49 B50 B54 B55 B56 B57 B58 B60 B61 B62 B63 B67 B72 B73 B75 B76 B77 B81 B82 | A1 A2 | A1 A2 B62 |
| Serum 8 | A1 A2 A23 A24 A29 A33 A34 A66 A68 A69 A74 A80 B7 B13 B18 B38 B41 B42 B45 B46 B48 B51 B52 B53 B54 B55 B56 B58 B59 B60 B61 B64 B65 B67 B73 B76 B77 B81 B82 | A1 A2 B51 | A1 A2 |
| Serum 9 | A2 A11 A24 A25 A26 A29 A33 A34 A43 A66 A68 A69 B38 B39 B51 B63 B64 B65 B77 B71 B18 B78 | A2 B51 | A2 |
| Serum 10 | A1 A24 A29 A36 A80 B44 B45 B57 B76 B82 | A1 | A1 |
| Serum 11 | A1 A3 A11 A25 A26 A29 A30 A31 A32 A33 A34 A36 A43 A66 A68 A74 A80 B8 B13 B37 B38 B39 B41 B42 B46 B47 B48 B54 B55 B56 B57 B58 B59 B61 B62 B63 B72 B73 B82 | A1 | A1 B62 |
| Serum 12 | A1 A2 A23 A24 A26 A29 A36 A80 B8 B44 B45 B57 B58 B82 | A1 A2 | A1 A2 |
| Serum 13 | A2 A23 A24 B7 B8 B13 B18 B27 B35 B39 B41 B42 B45 B47 B48 B50 B51 B53 B54 B55 B56 B57 B58 B60 B62 B70 B75 B76 B78 | A2 | A2 B62 B35 |

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