Online Supplemental Material

Platelet FcγRIIA-induced serotonin release exacerbates the severity of Transfusion-Related Acute Lung Injury in mice

Short title: FcγRIIA/CD32A expression in mice exacerbates TRALI.

Marie-Belle El Mdawar[1]*, Blandine Maître[1]*, Stéphanie Magnenat[1], Florian Tupin[1], Friederike Jönsson[2], Christian Gachet[1], Henri de la Salle[1#] and Beatrice Hechler[1#]
*M.-B.E.M. and B.M co-first authors. [#]H.dlS. and B.H co-last authors.

[1] Université de Strasbourg, INSERM, Etablissement Français du Sang (EFS)-Grand Est, BPPS UMR_S 1255, Fédération de Médecine Translationnelle de Strasbourg (FMTS), F-67000 Strasbourg, France; [2] Institut Pasteur, Department of Immunology, Unit of Antibodies in Therapy and Pathology, UMR INSERM U1222, Paris, France.

Corresponding author : Beatrice Hechler, UMR_S1255 INSERM, Université de Strasbourg, Etablissement Français du Sang-Grand Est, 10 rue Spielmann, BP 36, F-67065 Strasbourg Cedex, France. Tel. +33 3 88 21 25 25, Fax +33 3 88 21 25 21, email: beatrice.hechler@efs.sante.fr

Supplemental Methods

Reagents

Lipopolysaccharide (LPS, Escherichia coli, 055:B5, L28-80) was from Sigma-Aldrich; xylazine (Rompun) and ketamine (Imalgene 1,000) were from Bayer and Merial, respectively. Recombinant hirudin was from Transgene. Clodronate liposomes (clodronate 5 mg/mL) were from LIPOSOMA. The allogeneic anti-mouse MHC class I (H-2D^d/H-2K^d) mAb (clone 34-1-2S, IgG2a) and the anti-Ly6G/Ly-6C mAb (clone RB6-8C5) were from BioXcell. The anti-GPIbα (CD42b) mAb cocktail R300 was from Emfret and the FITC-conjugated rat anti-mouse P-selectin mAb (clone RB40.34) from BD Pharmingen. Rat anti-mouse Ly6G (clone 1A8) and PE-labeled anti-CD45 (clone 30-F11) were from Biolegend. A647-labeled goat anti-mouse IgG (H+L) was from Invitrogen and Cy5-labeled donkey anti-human IgG (H+L) from Jackson ImmunoResearch. A rat mAb against the extracellular domain of platelet GPIbβ (RAM.1) was produced and coupled to Alexa-647 in our laboratory. The following reagents were obtained through the NIH Tetramer Core Facility (tetramer.yerkes.emory.edu): H-2K^d IYSTVASSL monomer, H-2K^d SYIGSINNI monomer and H-2K^d IYSTVASSL Alexa-647.

Recombinant humanized 34-1-2S mAb (human IgG1 isoform, hIgG1-34-1-2S)

The 34-1-2S mAb was expressed as a human IgG1 Fc-mouse variable region chimera. Briefly, RNA from HB-79 hybriboma was reversed transcribed and the cDNAs encoding the variable regions of heavy and light chains of the 34-1-2S mAb were amplified by PCR [1]. The amplified fragments were inserted into two pUC19-based vectors expressing either the human IgG1 heavy chain cDNA or the light chain κ cDNA, respectively, generously provided by H. Mouquet [2]. The resulting chimeric chains were co-expressed, produced in HEK293 cells and, purified by Genescript.

Drugs, inhibitors and specific cell depletion

Fluoxetine was dissolved at 160 mg/L in drinking water and renewed twice a week for 3 weeks before TRALI experiments. Mice received an i.v. injection of sarpogrelate (1 mg/kg) or ABT-491 (1.25 mg/kg) 5 min before administration of the anti-MHC I mAb. Platelets or granulocytes were depleted by i.v. injection of an anti-GPIbα (CD42b) mAb cocktail (R300, 0.5 mg/kg in saline) or an anti-Ly6G/Ly-6C mAb (clone RB6-8C5, 0.5 mg/kg in saline), respectively, or as the negative control an isotype-matched antibody, 24 h prior to LPS administration. Monocytes and macrophages were depleted by i.v. injection of clodronate liposomes (2 mL/kg), 18 h after LPS administration, i.e., 6 h prior to injection of the anti-MHC I mAb, as previously described [3].

Protein content of bronchoalveolar lavages (BALs)

At the indicated time points after induction of TRALI, the animals were sacrificed, the left lungs were isolated by ligation (and used later for immunohistochemistry), the tracheas were exposed and cannulated and 800 µL of sterile PBS were injected into the right lungs. The bronchoalveolar lavages were recovered and centrifuged at 13,000 x g for 5 min at 4°C and the supernatants were stored at -80°C. The protein contents of BALs were quantified using a bicinchoninic acid assay (Thermo Fisher Scientific) according to the manufacturer's instructions with bovine serum albumin (BSA) as the standard.

ELISA assays

Blood was drawn from the abdominal aortas of anesthetized mice into a syringe containing EDTA anticoagulant (6 mM) 15 min after the induction of TRALI. Plasma concentrations of PF4, serotonin and MPO were determined using commercial kits (Quantikine® ELISA Mouse CXCL4/PF4 from R&D SYSTEMS™, Serotonin ELISA Fast Track from LDN® and MPO mouse ELISA Kit from Hycult Biotech).

Blood cell counts

The tails of anesthetized mice were severed, blood was collected into EDTA (6 mM) and blood cell counts were determined using a Scil Vet ABC automatic cell counter (Scil Animal Care Company) set to murine parameters.

Assessment of airway responsiveness

A FlexiVent system (SCIREQ Technologies, Canada) was used to measure the lung elastance (Ers) during TRALI. Briefly, mice were anesthetized i.p. with xylazine (30 mg/kg) and ketamine (150 mg/kg) and tracheotomized. An 18-gauge needle was inserted into the trachea and connected to a FlexiVent computer-controlled small animal ventilator. Stabilization of the baseline values was obtained within 4 min, after which the mAb hIgG1-34-1-2S (0.75 mg/kg) or saline was injected into the jugular vein. Respiratory parameters were measured using a FlexiVent protocol alternating normal ventilation and snapshot perturbations. At the end of the experiment, the mice were disconnected from the apparatus to ensure that they could still breathe without mechanical ventilation and then sacrificed.

Histology and immunohistochemistry

At the end of the experiments, the vasculature was flushed with 0.9% normal saline through a needle inserted into the left ventricle. The lungs of sacrificed mice were removed, fixed in 4% paraformaldehyde overnight and then washed in PBS and maintained in 10% and then 20% sucrose baths for one day each, before being snap-frozen in optimal cutting temperature (OCT) embedding medium. Sections (8 µm thick) were stained with hematoxylin and eosin for histological examination. The acute lung injury has been quantified in a blinded fashion, using a modified VILI histology scoring system as previously described [4] based on the four following parameters: 1) thickness of the alveolar walls/hyaline membranes, 2) leukocyte infiltration 3) hemorrhage and 4) alveolar congestion. Thrombi were counted in at least ten random fields under a light microscope (Leica DMLB, 200x resolution) by an investigator who was blinded to the various groups and the number of lung thrombi per square millimeter was calculated. Recruitment of platelets or neutrophils to the lungs was assessed on sections stained with a

rat mAb against the extracellular domain of GPIbβ (RAM.1) or with a rat anti-mouse Ly6G mAb (1A8), respectively, followed by the appropriate secondary goat anti-rat IgG conjugated to horseradish peroxidase. Color development was quantified after addition of 3',3'-diaminobenzidine. The area occupied by neutrophils or platelets was determined using Metamorph software, on images acquired with a Leica DMLB inverted microscope, in at least eight random fields ($150x10^3$ μm^2/field) on each section, by an investigator who was blinded to the various groups.

Flow cytometric analyses

The binding of recombinant hIgG1-34-1-2S or 34-1-2S to MHC I molecules was measured on the circulating $CD45^+$ leukocytes of BALB/c mice. Briefly, blood was collected into hirudin anticoagulant (100 U/mL) from the abdominal aorta of anesthetized mice. Fcγ receptors were blocked with FcR blocking reagent (Milteny Biotec), after which the cells were stained with 34-1-2S or hIgG1-34-1-2S followed by the appropriate secondary antibodies and then an anti-CD45 antibody. Red blood cells were lysed by addition of ACK lysis buffer before analysis of the samples in a Fortessa-X20 flow cytometer (BD Biosciences). $CD45^+$ leukocytes were analyzed using BD FACSDiva software and MHC I-positive cells were gated using MHC I-unstained cells (fluorescence minus one).

To evaluate platelet α-granule secretion, mouse whole blood was diluted in phosphate buffered saline (PBS) containing 0.35% human serum albumin, 100 U/mL hirudin and an FcR blocking reagent, prior to co-staining with a FITC-conjugated rat anti-mouse CD62P (P-selectin) mAb and an Alexa 647-conjugated anti-GPIbβ mAb (RAM.1). Samples were analyzed using a Gallios flow cytometer and its accompanying Kalusa software (Beckman Coulter). Scattering and fluorescence intensity from 10,000 cells were collected with a logarithmic gain and results were expressed as the percentage of positive events.

Bio-layer interferometry

Measurements were performed at 25°C on Octet RED394 (ForteBio) using streptavidin-coated biosensors (ForteBio). After conditioning, the biosensors were saturated with biotinylated MHC I monomers (500 nM in PBS) for 10 min and then incubated in serial dilutions of antibodies (0 to 20 nM) in PBS-BSA (1 mg/mL) for 30 min (association). Dissociation was measured after transfer of the antibody-bound sensors into PBS-BSA (1 mg/mL) for 30 min. Background signals were subtracted and the binding rates Kon (association rate) and Koff (dissociation rate) and dissociation equilibrium constant Kd were determined with the Octet data analysis software. Control experiments confirmed the absence of non-specific antibody binding to the empty sensors.

Immunoglobulin binding assays

Stably transfected CHO-K1 expressing either FLAG-tagged $CD32A_{R131}$ or mouse FcγRs were cultured as described [5,6]. IgG immune complexes were formed by incubating 1 µg/mL mIgG2a-34-1-2S or hIgG1-34-1-2S with 0.25 µg/mL A647-labeled MHC I H-$2k^d$ tetramer in MACS buffer (PBS 0.05% BSA, 2 µM EDTA pH7.4) for 30 min at 37°C. CHO transfectants (2×10^5 cells) were incubated with IgG complexes for 30 min at 4°C, washed and analyzed by flow cytometry (MACSQuant16, Miltenyi Biotec). Alternatively, cells were first incubated with 1 µg/mL mIgG2a-34-1-2S or hIgG1-34-1-2S for 30 min at 4°C, washed, and then incubated with 0.25 µg/mL A647-labeled MHC I H-$2k^d$ tetramer prior to washing and analysis to reveal binding of non-aggregated IgG. Transfection levels were determined using an APC-labelled anti-Flag antibody (Miltenyi Biotec).

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Supplemental Figure Legends

Supplemental Table 1: Baseline blood cell counts in CD32A$^-$ and CD32A$^+$ mice.

Blood cell counts were determined using a Scil Vet ABC automatic cell counter (Scil Animal Care Company) set to murine parameters. Results are presented as the mean ± SEM (n=30).

Supplemental Figure 1: The original mouse mAb mIgG2a-34-1-2S and the chimeric human hIgG1-34-1-2S bind similarly to MHC I.

A. Mouse IgG2a-34-1-2S and hIgG1-34-1-2S bind to H-2K^d MHC I with comparable affinity. Association and dissociation profiles of the original mouse mAb IgG2a-34-1-2S and the chimeric human/mouse mAb hIgG1-34-1-2S on H-2K^d MHC I were determined using Bio-Layer Interferometry (BLI). Streptavidin-coated sensors were saturated with biotinylated H-2K^d MHC I monomers and immersed in twofold serial dilutions of antibodies ranging from 20 to 0.32 nM. Panels show the BLI shift during the association and dissociation steps (separated by the dotted line). K_D for both antibodies was calculated as K_{off}/K_{on}. Representative curves from 4 experiments (using two different MHC monomers) are shown. **B.** Mouse IgG2a-34-1-2S and chimeric hIgG1-34-1-2S bind similarly to CD45$^+$ murine leukocytes *in vitro*. Blood cells were stained with an anti-MHC I mAb (IgG2a-34-1-2S or hIgG1-34-1-2S) and an anti-CD45 mAb as described in the Methods. CD45$^+$ leukocytes were analyzed by flow cytometry (10,000 events) and MHC I-stained and -unstained cells were compared (fluorescence minus one, FMO). **C.** Mouse IgG2a-34-1-2S and chimeric hIgG1-34-1-2S bind similarly to CD32A$_{R131}$ and mouse FcγRs. Flow cytometric analysis of the binding of preformed immune complexes of chimeric hIgG1-34-1-2S (red) and mouse IgG2a-34-1-2S (orange) or non-aggregated IgG (dark and bright blue, respectively) to CHO transfectants expressing a single Flag-tagged FcγR (mouse FcγRI-IV or human CD32A$_{R131}$). Unstained cells (grey) and tetramer staining alone (black dashed line) indicate background fluorescence, Flag-staining (dark grey) informs on receptor expression level. Representative of three independent experiments.

Supplemental Figure 2: The presence of a few occlusive thrombi in the pulmonary microcirculation of CD32A$^+$ and CD32A$^-$ mice.

LPS-sensitized (0.1 mg/kg, i.p.) CD32A$^-$ or CD32A$^+$ mice were injected with the mAb hIgG1-34-1-2S (1.5 mg/kg, i.v.) or vehicle and examined 15 min later. Left: Number of thrombi per unit surface area in the lungs of CD32A$^-$ and CD32A$^+$ mice (n=3). Right: Representative H&E-stained lung sections from CD32A$^-$ and CD32A$^+$ mice showing the presence of a few occlusive thrombi (arrowhead) (Scale bar, 100 µm). Results are presented as the mean ± SEM.

Supplemental Figure 3: Confirmation of neutrophil depletion before the induction of TRALI.

Kinetics of circulating blood neutrophils (**A**) and monocytes (**B**) following injection of the Gr-1 mAb RB6-8C5 or an rIgG2bk isotype-matched control into CD32A$^-$ or CD32A$^+$ mice. RB6-8C5 or rIgG2bk (0.5 mg/kg, i.v.) was administered on day 1 (D1), 24 h before LPS (0.1 mg/kg, i.p.) (D2) and TRALI was induced on D3. Results are presented as the mean ± SEM (n=3-4).

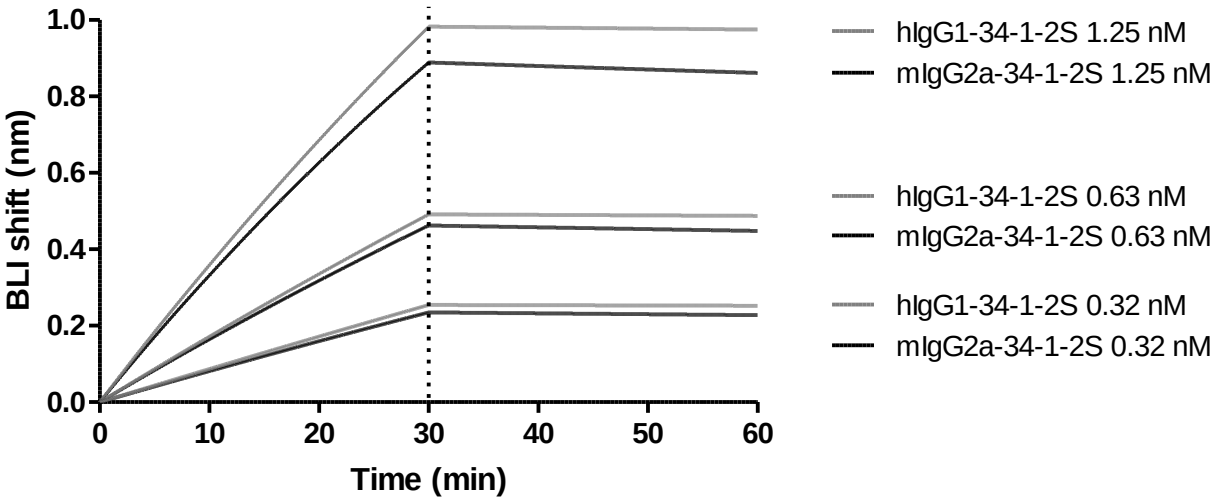
Supplemental Figure 4: Confirmation of platelet depletion before the induction of TRALI.

Kinetics of platelet depletion following injection of the anti-GPIbα (CD42b) mAb cocktail R300 or an rIgG2a isotype-matched control into CD32A$^-$ or CD32A$^+$ mice. R300 or rIgG2a (0.5 mg/kg, i.v.) was administered on D1, 24 h before LPS (0.1 mg/kg, i.p.) (D2) and TRALI was induced on D3. Results are presented as the mean ± SEM (n≥17).

Supplemental Table 1

	CD32A$^-$	**CD32A$^+$**
White blood cells (x10^3/µL)	6.60 ± 0.36	6.68 ± 0.38
Neutrophils (x10^3/µL)	1.82 ± 0.22	1.61 ± 0.13
Lymphocytes (x10^3/µL)	4.54 ± 0.34	4.86 ± 0.31
Monocytes (x10^3/µL)	0.20 ± 0.02	0.22 ± 0.02
Eosinophils (x10^3/µL)	0.050 ± 0.003	0.055 ± 0.004
Platelets (x10^3/µL)	1108 ± 18	1109 ± 16
Red blood cells (x10^5/mL)	10.08 ± 0.09	10.35 ± 0.09
Hemoglobin (g/dL)	16.9 ± 0.1	17.2 ± 0.1

A.



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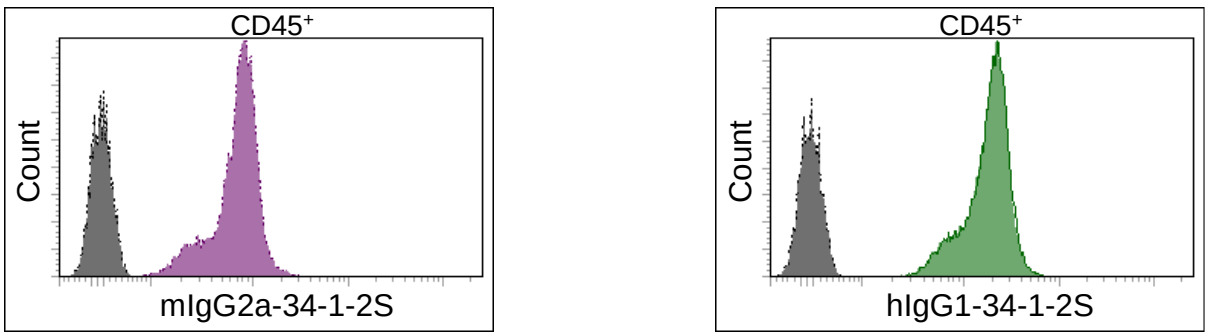
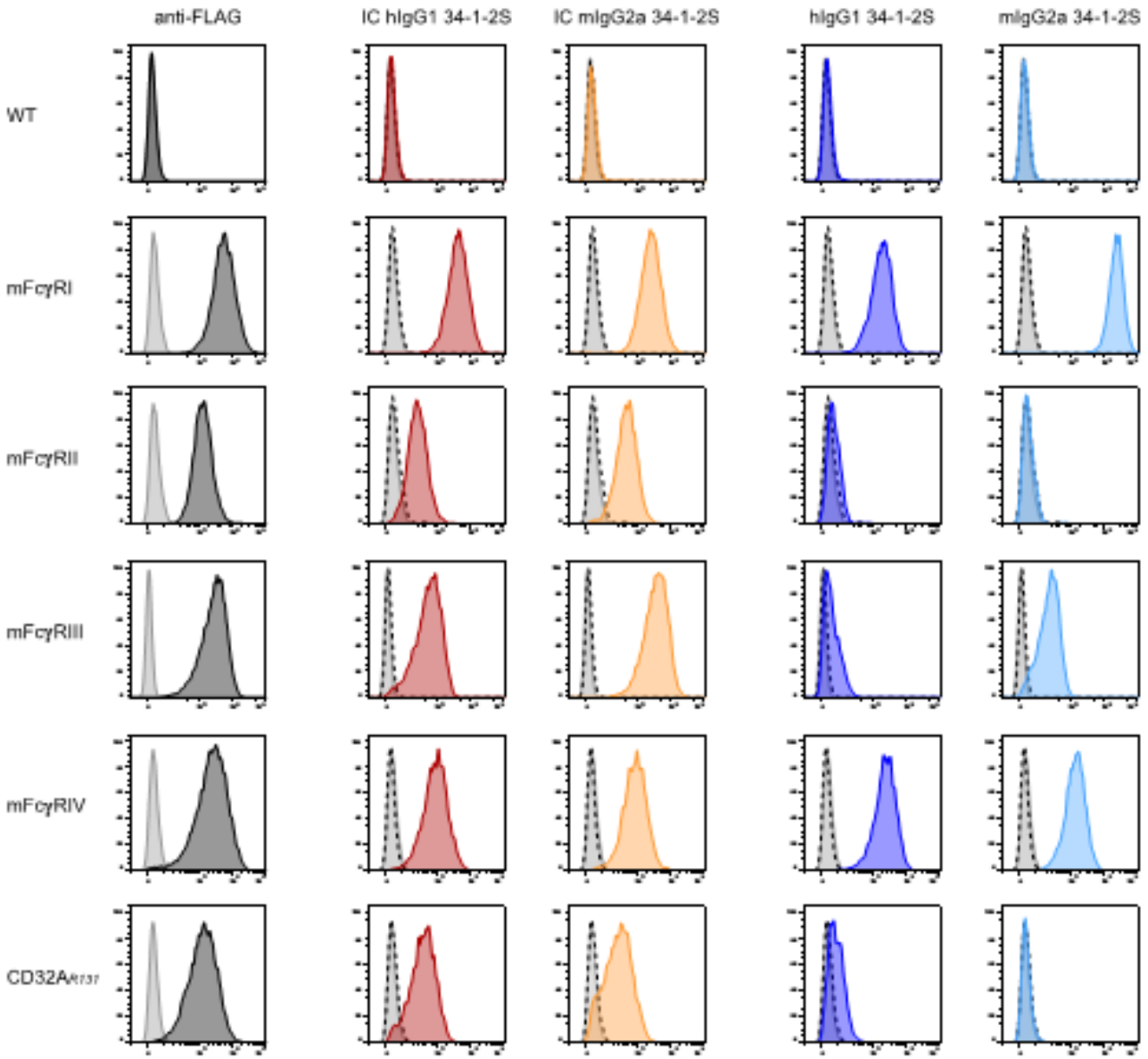
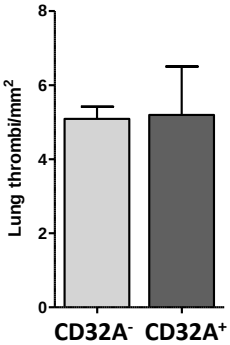


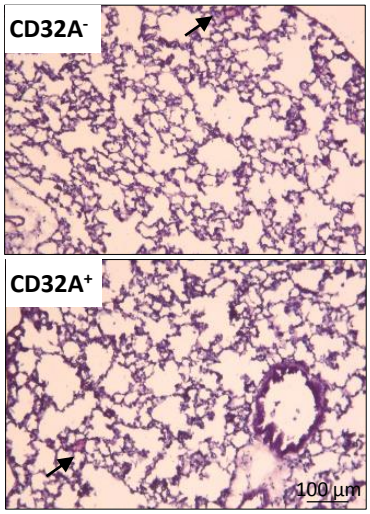
Supplemental Figure 1A,B

C.



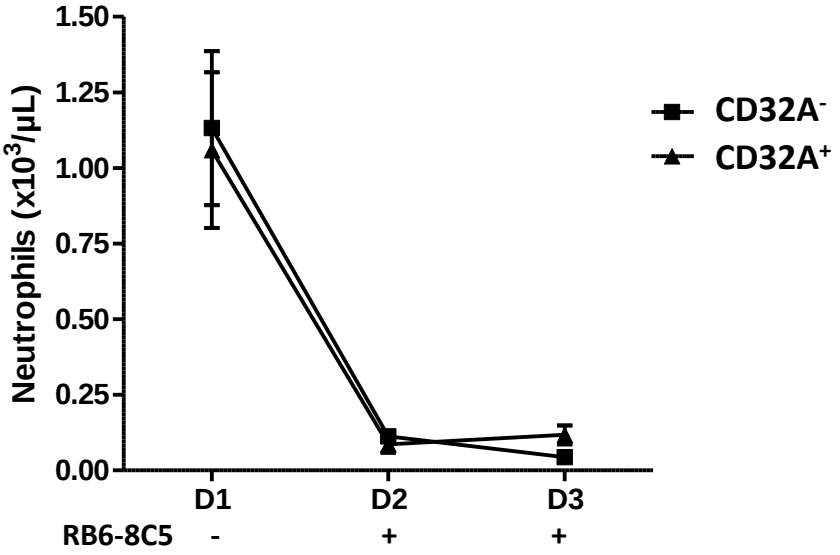
Supplemental Figure 1C





Supplemental Figure 2

A.



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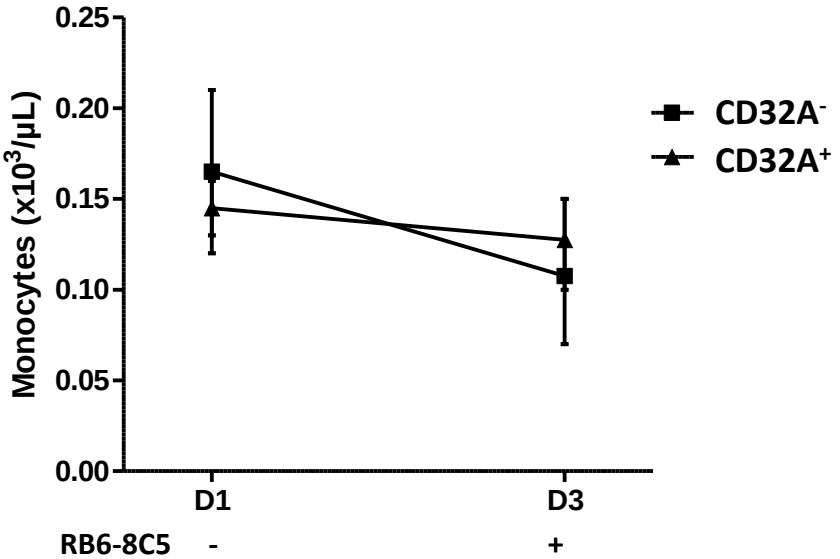
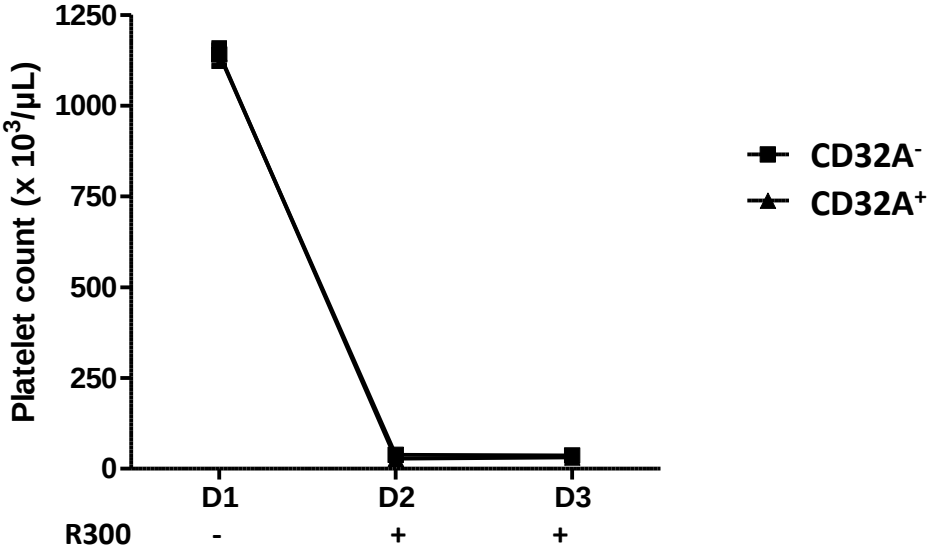


Supplemental Figure 3



Supplemental Figure 4