# Clinical severity in adult warm autoimmune hemolytic anemia and its relationship to antibody specificity

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SUPPLEMENTAL MATERIAL

PATIENTS & METHODS

**Patients:**

This was a single-center cohort study performed in the Department of Internal Medicine at Henri Mondor University Hospital (Creteil, France), a tertiary-care national referral center for adult autoimmune cytopenias. We included data for all consecutive patients with a definite diagnosis of wAIHA between 2011 and 2015 who fulfilled the following eligibility criteria: (1) age 16 years at diagnosis; (2) diagnosis of AIHA defined by a hemoglobin (Hb) level of 11 g/dL with features of hemolysis (low haptoglobin and/or elevated lactate dehydrogenase [LDH] level and/or elevated bilirubin level) and a positive direct antiglobulin test (DAT) result with an IgG or $IgG_1$ C3d pattern; and (3) the absence of any other cause of acquired or hereditary hemolytic anemia. Each treatment was considered as a treatment line: steroids, intravenous immunoglobulin, immunosuppressive therapy, rituximab and splenectomy.

**Autoantibody characteristics and specificity**

Investigations on patient blood samples, elution, and characterization of the autoAbs included direct antiglobulin tests (DATs), antibody screening tests, and the search for antibodies in serum (indirect antiglobulin tests, IATs). Acidic elution of the autoAbs was performed on washed, coated RBCs obtained from the patients. All samples were collected during the period between presentation and splenectomy. This research was performed in accordance with the ethical recommendations of the statement of Helsinki and approved by our institutional ethics committee.

*Routine explorations on patient blood samples.*

Agglutination reactions, performed by the gel-test microcolumn technique (Micro-Typing system, DiaMed, a division of Biorad, Cressier, Switzerland), were used for serological testing, according to the manufacturer's recommendations. Cards containing monospecific rabbit anti-human IgG, C3b, C3d, IgM, or IgA antibodies were used for the DAT and IAT, and to screen sera and eluates. DATs were performed with a polyclonal anti-IgG and a monoclonal anti-C3d reagent (DC-Screening II). IgG subclasses were determined using DAT $IgG_1$/$IgG_3$ cards.

*Elution and characterization of the autoantibodies bound to patient RBCs.*

Antibodies were eluted from the washed patient' RBCs using an acid elution technique (Elu-kit II, Immucor Inc, Norcross, GA) as recommended by the manufacturer. The resulting eluates (1mL total volume) were used to determine the class, specificity, titer, and thermal range of the eluted Ig autoAbs, as fully described in a previous study of our group.[1] They were used immediately or stored at -80°C for further studies. The specificity of eluted autoAbs was determined using rare RBCs lacking specific protein antigens with high incidence or normal RBCs treated with various enzyme-proteases (ie. trypsin, chymotrypsin, papain, ficin and pronase) to depict their pattern of reactivity after treatment. Agglutination-blocking tests were then performed with a panel of murine IgG monoclonal antibodies (mAbs) produced in-house (St-Louis Hospital, Paris, France) and untreated or treated RBCs by incubation with two-fold serially diluted eluates.[1-3] Reactions were carried out at +37°C, for 30 min, on neutral gel cards and IAT reactions were detected with polyclonal anti-IgG antiglobulin serum (Biotest, Buc, France). The class and sub-class of autoAbs found in the eluate was determined by DAT in column gel-cards, as previously described. The thermal range was determined by titration tests against normal RBCs at +37°C and +20°C. The titer of the eluates was defined as the last dilution giving a positive reaction. Eluate samples were diluted in phosphate buffer saline (PBS), pH 7.4, containing 1.5% bovine serum albumin (BSA) to a final titer between 8 and 128 for further investigation.

### *In vitro* cellular investigations

Experiments were performed with peripheral blood mononuclear cells (PBMCs) isolated from healthy donor blood samples collected in $Na^+$-heparinized tubes. The normal RBCs used in the tests were obtained from healthy, RhD-positive donor blood samples collected in EDTA tubes, centrifuged, and washed three times in HBSS. In each case, autoAbs were obtained after acid elution of patient RBCs.

*Phagocytosis of RBCs (monocyte monolayer assay, MMA)*

Normal PBMCs from healthy RhD-positive donors were separated on a Ficoll density gradient (Eurobio, Les Ulis, France), washed twice in HBSS and resuspended in RPMI-1640 medium (Invitrogen Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS). Aliquots of 0.5ml ($2x10^6$ cells/ml) of PBMCs were dispensed into an eight-chamber culture slide (Labtek, BD Falcon, San Jose, CA) and incubated for 45 min at +37°C. In parallel, washed RBCs from the same donors were sensitized by incubation with eluted autoAbs for 30 min at +37°C. A mouse mAb of the IgG-type HM16 directed against human RhD (Diagast, Loos, France) or a humanized polyclonal anti-RhD IgG (rhophylac; 100µg/mL; LFB Biotechnologies, Courtabœuf, France) were used to sensitize undiluted RBCs as positive controls. Once the monocytes adhered, the culture chambers were washed with PBS. The adherent cells were overlaid with the RBC suspension (5% hematocrit in RPMI-1640 +10% FCS) and incubated for a further 2h at +37°C. At the end of the incubation period, unbound RBCs were removed by rinsing with PBS. The slides were fixed with methanol, stained by the May-Grünwald Giemsa method, and observed under a light microscope. For each set of conditions, 200 monocytes from three to four different areas of the slide were counted (by two operators each time) to assess RBC phagocytosis and adhesion, reflecting monocyte reactivity (%). Mean values > 5% were considered to be positive.[1,4]

*ADCC test*.

The capacity of eluted autoantibodies to trigger Ab-dependent cellular cytotoxicity (ADCC) by

normal PBMC subsets was evaluated *in vitro* on the basis of transient CD107a protein exposure at the cell surface.[1,5] PBMCs obtained from healthy, RhD-positive donors, adjusted to $10^7$ cells/ml, were used as effector cells. Washed RBCs, obtained from the same donors, were sensitized with a human polyclonal anti-RhD IgG antibody (rhophylac; LFB) or eluted autoantibodies from the eluates obtained from the patient samples (n=29). The sensitized RBCs were used as the target cells. An effector/target (E/T) ratio of 1:1 was used in all experiments. We added 5µL of PE-Cy5-conjugated H4A3 anti-CD107a mAb (BD Biosciences, Le Pont de Claix, France) for each condition before incubation. PBMCs and RBCs were incubated together for a total of 5 h at +37°C/ 5% $CO_2$. The secretion inhibitor monensin (2µM, Sigma-Aldrich, St. Louis, MO, USA) was added after 1h. At the end of the incubation period, cells were washed in PBS, stained with the various mAbs (FITC-conjugated SK7 anti-CD3; PE-conjugated SK1 anti-CD8, MY31 anti-CD56; BD Biosciences), and CD107a expression estimated on gated cellular events. A control sample without target cells was included in each experiment for the detection of spontaneous degranulation. Data were collected by flow cytometry on a FACS Canto 2 (BD) and analyzed using Diva 6 software.

*Erythro-trogocytosis detection*.

PBMCs and RBCs from donors were used to measure trogocytosis (i.e. active membrane transfer between sensitized RBCs and various PBMC populations) as previously described.[1,6] RBCs obtained from the same healthy RhD-positive donors were stained with the lipophilic green-emitting dye PKH-67 (Sigma-Aldrich), according to the manufacturer's instructions. They were sensitized with monoclonal HM16 anti-human RhD IgG (Diagast) or humanized polyclonal anti-RhD IgG (rhophylac; 100µg/mL; LFB) for the positive controls, or eluted autoAbs. The RBCs, which served as targets, were incubated with PBMCs, at a RBC:PBMC ratio of 2:1, for 60 min at +37°C. The cells were then washed with 2mM EDTA in PBS to disrupt cellular synapses. PBMC subsets were stained with specific monoclonal antibodies for 30 min at +4°C and flow cytometric analysis was then carried out. We investigated the involvement of the FcR in Ig-

induced RBC trogocytosis (blocking test) by treating PBMCs with a polyspecific pan FcR-blocking reagent (Fc-Blocker, Miltenyi Biotec), as previously described by our group,[1] which efficiently blocks the binding of microbeads or antibodies to the Fc receptor of FcR-expressing cells, including monocytes (see also supplemental figure S4). Briefly, PBMCs in 90µL PBS+0.2% BSA were incubated twice for 10 min each with saturating concentrations of Fc-Blocker at +4°C, as indicated by the manufacturer (20µL for $10^7$ cells); blocking experiments using a 5-fold saturating concentration of Fc-Blocker gave similar results (data not shown). Treated PBMCs were washed, stained with the various mAbs (phycoerythrin (PE)-conjugated mAbs SK7 anti-CD3, SK3 anti-CD4, SK1 anti-CD8, MY31 anti-CD56, and MΦP9 anti-CD14; BD Biosciences) and tested for erythro-trogocytosis. Data were collected and analyzed by flow cytometry. Synaptic transfer was assessed as the acquisition of PKH-67 fluorescence from PKH-67-labeled RBCs by the various leukocyte subset populations. This acquisition led to an increase in mean fluorescence intensity (MFI) for PKH-67. For the detection of the spontaneous PKH-67 fluorescence released from untreated RBCs and the transfer of this fluorescence to leukocytes, a T0 control sample (″instant contact″) was included in each experiment.

**Statistical analyses**

Quantitative data are expressed as the arithmetic mean ± standard deviation (SD), and categorical data as numbers with percentages. Statistical analyses were performed using Student *t* tests, Mann-Whitney *U* tests, or analyses of variance (ANOVA), with GraphPad Prism software version 5.03 (La Jolla, CA, USA). Values of $p<0.05$ were considered to be statistically significant.

SUPPLEMENTAL FIGURES AND TABLES

**Supplemental table S1:** Classes and subclasses of autoantibodies from the samples of the cohort

**Supplemental table S2:** Clinical data and immuno-hematological parameters of the patients

**Supplemental fig S1: $CD14^+$-mediated trogocytosis with autoAb-sensitized RBCs: correlations with Hb levels and phagocytic index; inhibition index with a polyspecific FcR Blocker.**

(A) Correlation between the $CD14^+$ erythro-trogocytosis index and Hb levels ($R^2$=0.1489; *p*=0.0840). (B) Correlation between the trogocytosis index and the phagocytic index, both obtained with $CD14^+$ monocytes ($R^2$=0.009773; *p*=0.6616). (C) Trogocytosis inhibition index with the Fc-blocker: histograms show the inhibition of the fluorescence transferred to $CD14^+$ monocytes. The value obtained for sensitized RBCs incubated with untreated monocytes represent 100% for each autoAb specificity; bars indicate the mean ± standard deviation.

**Supplemental fig S2: Inhibition of FcγR-mediated trogocytosis of $CD14^+$ after treatment with the blocking antibodies CD64 and CD16 or the FcR blocker.**

(A) Erythro-trogocytosis dot plots obtained by flow cytometry and expressed as raw data (PKH-67 $CD14^+$ MFI) with normal RBCs sensitized with eluted autoAbs from AIHA patients and then challenged 60 minutes with $CD14^+$ monocytes. Monocytes were treated or not with specific anti-FcγR blocking antibodies (CD64/FcγRI and CD16/FcγRIII) or the polyspecific FcR-blocking mixture reagent (Fc Blocker) before co-incubation. Results are from a representative experiment. (B) Raw data (MFI) for erythro-trogocytosis shown as the means ± SD from at least three independent experiments.

**Supplemental fig S3: *In vitro* ADCC index as a function of autoAb specificity.**

(A) Cytotoxicity index obtained by flow cytometry (CD107a MFI on NK cells) for NK cells challenged with RBCs sensitized or not with eluates, as a function of autoAb specificity. PBMCs were the effector cells and RBCs the target cells; they were mixed and incubated together for five hours with a monoclonal anti-CD107a IgG. CD107a expression was then assayed on gated cellular events corresponding to NK cells ($CD3^-$ $CD16/56^+$ events). Data were collected on a BD Canto 2 flow cytometer. The baseline cutoff was determined for the experiment (MFI obtained for untreated RBCs, NT: 260±24). Results are shown as the means ± SD from at least three independent experiments. (B) Correlation between Hb levels and ADCC ($R^2$=0.024; $p$=0.463).

**Supplemental fig S4: Validation experiments for the inhibition of FcγR-mediated trogocytosis of $CD14^+$ monocytes after treatment with CD64 and CD16 or the FcR blocker.**

(A) Erythro-trogocytosis dot plots obtained by flow cytometry and expressed as raw data (PKH-67 $CD14^+$ MFI) with normal RBCs sensitized with a commercial monoclonal anti-RhD antibody (MH16) and then challenged 60 minutes with $CD14^+$ monocytes. Before co-incubations, monocytes were treated or not with anti-FcγR blocking antibodies (CD64/FcγRI and CD16/FcγRIII) or the polyspecific pan FcR-blocking reagent, Fc Blocker. Results are from a representative experiment. (B) Erythro-trogocytosis raw data (MFI) reported as the means ± SD from at least three independent experiments.

SUPPLEMENTAL REFERENCES

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**Supplemental table S1:** Classes and subclasses of autoantibodies from the samples of the cohort (n=52)

| **IgG positive DATs** | | | | | | |
|---|---|---|---|---|---|---|
| $IgG_1$+ | | $IgG_2$+ or $IgG_4$+ (suspected) | | IgM+ or C3+ | C3d+ alone | Neg DAT |
| C3+ | C3- | C3+ | C3- | | | |
| **23** | **13** | **2** | **6** | **1** | **3** | **4** |
| mixed with: | mixed with: | | mixed with: | | | |
| • $IgG_3$: 3 cases | • $IgG_3$: 3 cases | | • IgA: 1 case | | | |
| • IgA: 3 cases | • IgA: 1 case | | | | | |
| • IgM: 4 cases | | | | | | |
| • IgA/ IgM: 2 cases | | | | | | |
| • $IgG_3$/ IgM: 2 cases | | | | | | |
| **69.2 %** | | **15.4 %** | | **1.9 %** | **5.8 %** | **7.7 %** |

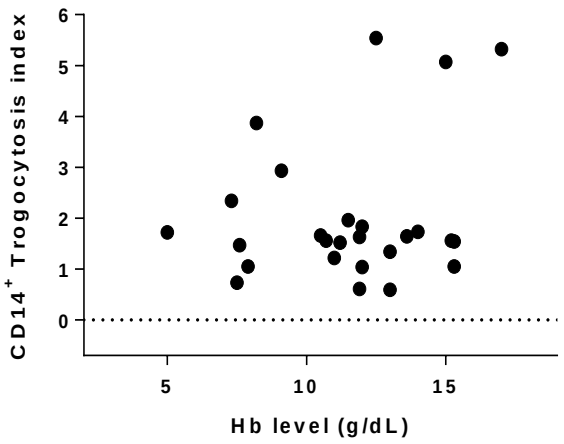
**Supplemental table S2:** Clinical data and immune-hematological parameters of the patients (n=29)

| *Patient code* | Sex | Age | DAT results | Indication | Evolution period (months) | Specificity | Primary / secondary AIHA | If secondary AIHA, associated with | Nadir Hb | Hb | IVIG (Yes/No) | RTX (Yes/No) | Actual treatment | Phagocytic activity (score) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***1/005.1*** | F | 43 | IgG + / IgA + | post-splenectomy | 19 | Band 3 | Primary | | 4,2 | 13,6 | No | Yes | none | 1+ |
| ***1/019.1*** | F | 23 | IgG + / C3d + | remission phase | 27 | Band 3 | Primary | | 5,3 | 13 | No | No | none | 1+ |
| ***1/022.1*** | M | 43 | IgG + (IgG1) | recent diagnosis, initial phase | * | Band 3 | Secondary | Hepatitis C | 5,7 | 7,3 | No | No | none | 2+ |
| ***1/023.1*** | F | 56 | IgG + (IgG1, IgG3) / C3 + | remission phase | 120 | Band 3 | Secondary | Hepatitis C | 7.9 | 17 | No | Yes | none | 2+ |
| ***1/028.1*** | F | 76 | IgG + / C3d + | relapse after initial remission | 22 | Band 3 | Primary | | 6,5 | 11 | No | No | Prednisone 8mg per day | 4+ |
| ***1/032.1*** | M | 79 | IgG + (IgG1) | remission phase | 2 | Band 3 | Primary | | 8 | 15,2 | No | No | none | 4+ |
| ***3/003.1*** | F | 46 | IgG + (IgG1) / IgA + | initial phase | * | Band 3 | Secondary | Hypothyroidism | 6,5 | 9,1 | No | Yes | | 5+ |
| ***3/004.1*** | F | 27 | IgG + (IgG1, IgG3) / IgA + / IgM + / C3 + | recent diagnosis | 1 | Band 3 | Primary | | 7,4 | 7,4 | No | Yes | | |
| ***1/002.1*** | F | 53 | IgG + (IgG1, IgG3) / C3d + | remission phase | 53 | unknown | Secondary | Splenic lymphoma | 5,6 | ǂ | No | Yes | Prednisone 8mg per day | **5+** |
| ***1/016.1*** | F | 80 | IgG + (IgG1) / IgM + / C3d + | low Hb level, even with Prednisone | 9 | unknown | Primary | | 4,7 | 8,2 | No | Yes | none | **2+** |
| ***1/031.1*** | M | 73 | IgG + (IgG1) / IgA + / C3d + | remission phase | 1 | unknown | Primary | | 7,1 | 13 | No | Yes | Corticoïds + Azathioprine | **4+** |
| ***1/006.1*** | F | 77 | IgG + (IgG1) | remission phase | 124 | GPA | Secondary | B cells lymphoid hemopathy | 4,9 | 15,3 | No | Yes | none | **4+** |
| ***1/010.1*** | F | 63 | IgG + (IgG1) / IgA + / C3 + | remission phase | 9 | GPA | Primary | | 6,1 | 11,5 | No | Yes | Danazol | **1+** |
| ***1/011.1*** | M | 47 | IgG + (IgG1, IgG3) / C3 + | remission phase | 36 | GPA | Primary | | 2,4 | 12 | No | Yes | Prednisone | **5+** |
| ***1/013.1*** | M | 32 | IgG + (IgG1) / IgM + / C3 + | remission phase | 59 | GPA | Primary | | 7,1 | 10,5 | No | Yes | Prednisone + Azathioprine | **5+** |
| ***1/017.1*** | F | 32 | IgG + (IgG1) / C3d + | remission phase | 11 | GPA | Secondary | Lupus | 3,4 | 11,9 | No | No | Prednisone | **1+** |
| ***1/026.1*** | M | 32 | IgG + (IgG1, IgG3) / IgM + / C3d + | initial phase | 1 | GPA | Secondary | Lupus and papillary sarcoma | 11 | 12,5 | No | No | Prednisone | **3+** |
| ***1/029.1*** | F | 45 | IgG + (IgG1) / IgA + / C3d + | initial phase | 5 | GPA | Secondary | Mixed connectivity | 6 | 10,7 | No | No | Prednisone | **4+** |
| ***1/001.1*** | F | 80 | IgG + | remission phase | 8 | RH | Primary | | 8,6 | 11,9 | No | No | none | **1+** |
| ***1/004.1*** | M | 37 | IgG + (IgG1) | low Hb level | 96 | RH | Primary | | 3,3 | 7,6 | No | Yes | | **2+** |
| ***1/007.1*** | F | 77 | IgG + (IgG1) | remission phase | 30 | RH | Secondary | CLL | 8,5 | 15,3 | No | Yes | | **2+** |
| ***1/009.1*** | F | 80 | IgG + (IgG1, IgG3) / C3 + | remission phase | 132 | RH | Primary | | 9,1 | 14 | No | No | Prednisone + Azathioprine | **1+** |
| ***1/018.1*** | M | 62 | IgG + (IgG1) | remission phase | 14 | RH | Primary | | 7,4 | 15 | No | No | none | **1+** |
| ***1/024.1*** | M | 85 | IgG + (IgG1, IgG3) / C3 + | recent diagnosis, initial phase | * | RH | Secondary | CLL | 6,4 | 6,4 | No | No | | |
| ***1/030.1*** | M | 74 | IgG + (IgG1) / IgA + / C3d + | initial phase | * | RH | Primary | | 7,9 | 7,9 | No | No | none | **5+** |
| ***3/001.1*** | F | 78 | IgG + (IgG1) | before rituximab | * | RH | Primary | | 6,3 | 11,2 | No | | | **5+** |
| ***3/002.1*** | M | 89 | IgG + (IgG1, IgG3) | recent diagnosis, initial phase | * | RH | Primary | | 7,5 | 7,5 | No | | | **2+** |
| ***1/033.1*** | M | 56 | IgG + (IgG1) | remission phase | 1 | RH | Primary | | 10,6 | 12 | No | No | none | |
| ***1/035.1*** | F | 60 | IgG + (IgG1) | before Rituximab | 14 | RH | Primary | | 6 | 10,8 | No | No | | |

M: male; F:female; Hb: hemoglobin; Ig: immunoglobulin; C3: complement fraction 3; CLL: chronic lymphocytic leukemia; GPA: Glycophorin A; RH: Rhesus; IVIG: intravenous immunoglobulin ; RTX: rituximab, anti-CD20. ǂ unable to be measured; * recent diagnosis, less than 1 month.
Phagocytic activity score is: [3-7%[ =**1+**; [7-15%[ = **2+**; [15-30%[ = **3+**; [30-49%[ = **4+**; [50-85%[ = **5+**

Supplemental figure S1

A

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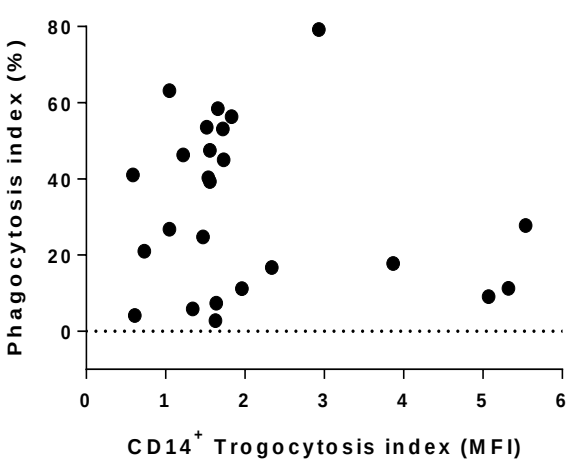


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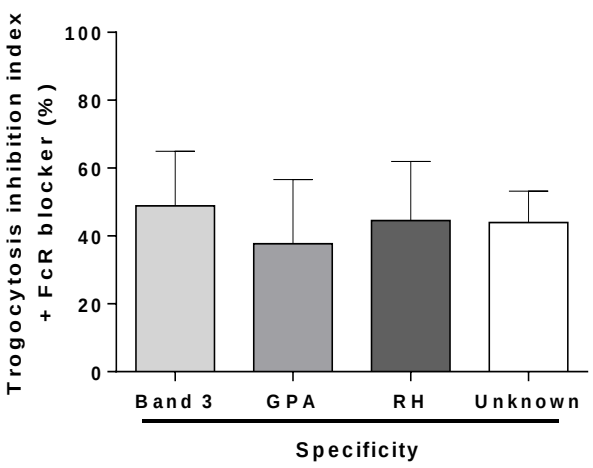
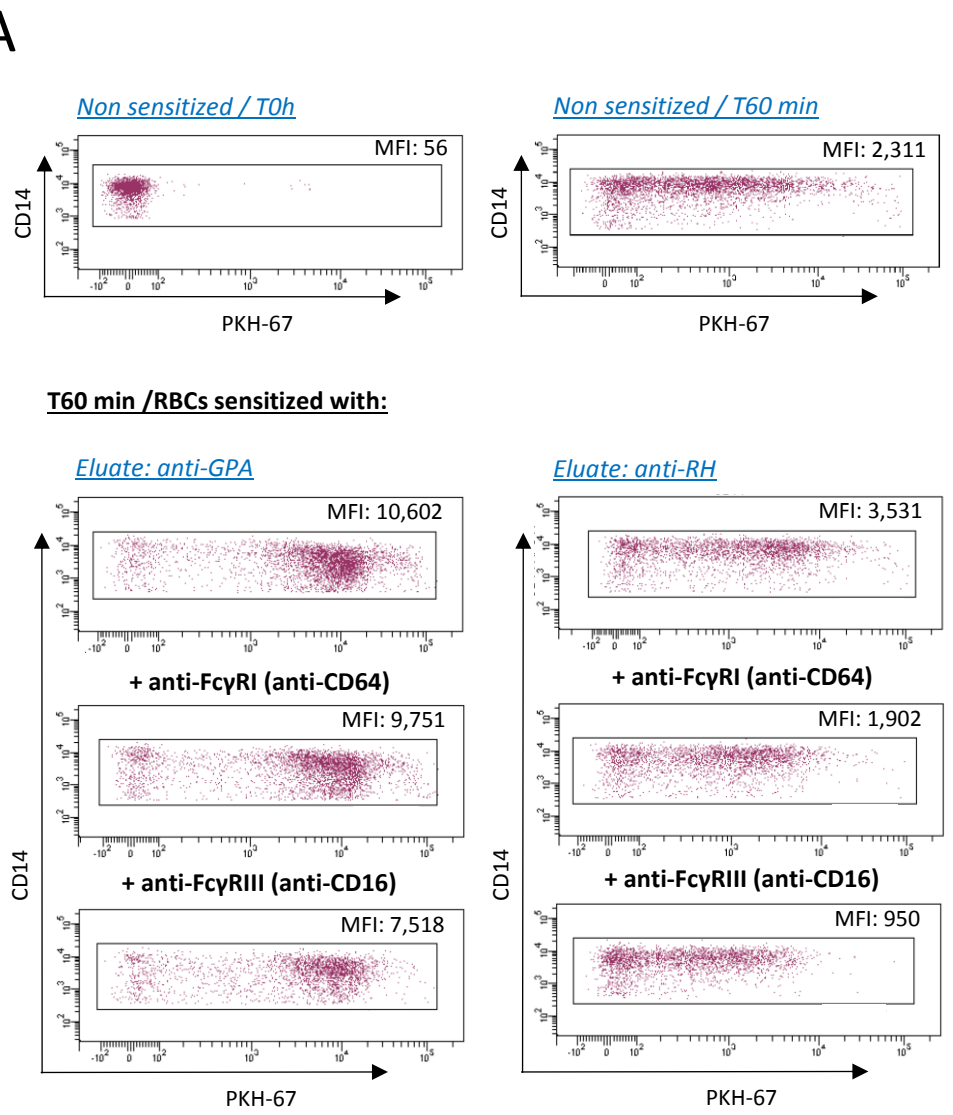
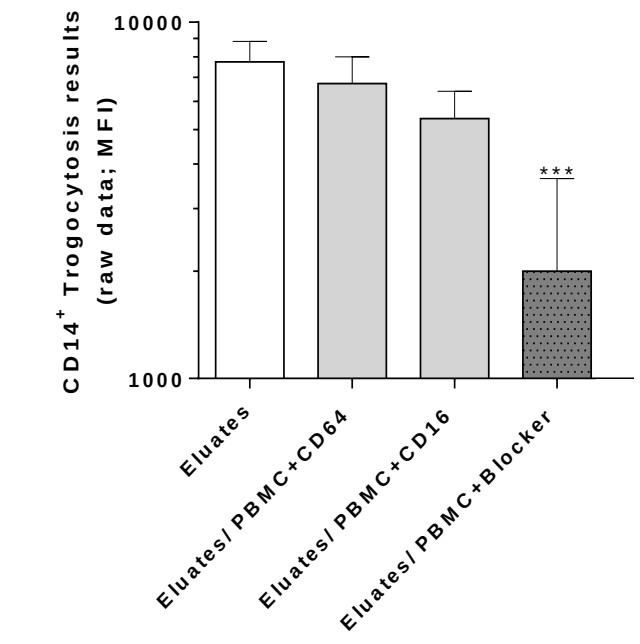


Supplemental figure S2



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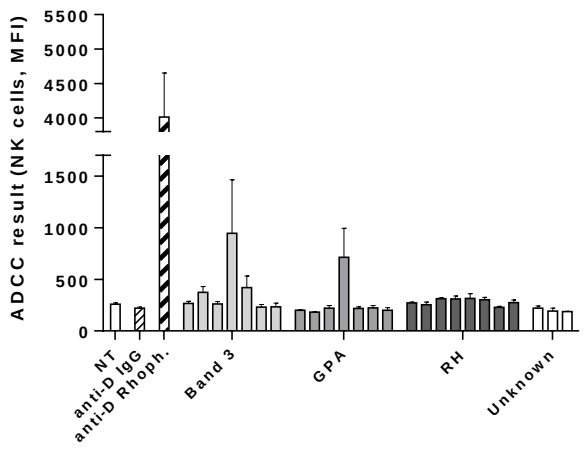


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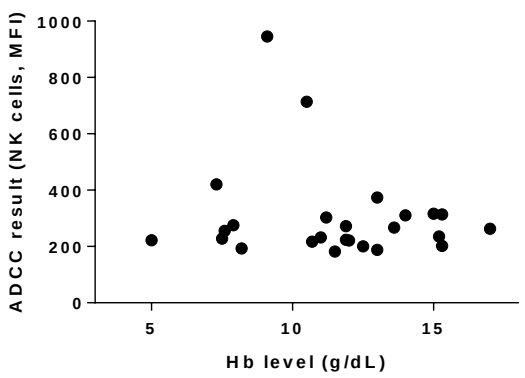

Supplemental figure S4

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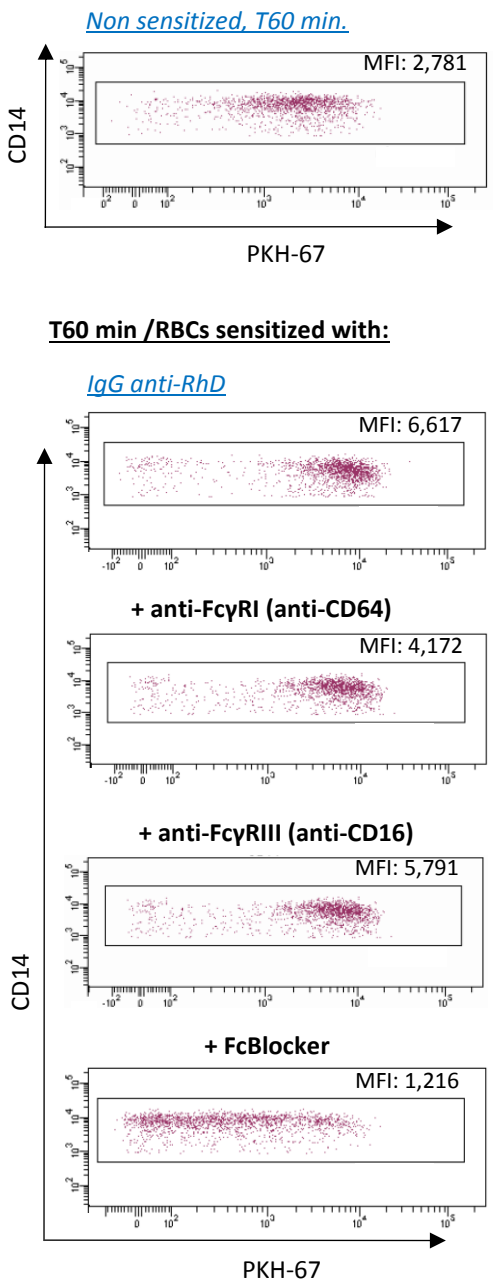


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