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Recommendations for the use of NOD/SCID mouse model in autoimmune- and drug-induced thrombocytopenia: communication from the SSC of the ISTH

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Brief introduction

Human platelet survival studies have been hampered due to the lack of a suitable animal model. Transfusion of human platelets into immune competent animals leads to the rapid destruction of these platelets by naturally occurring xenoantibodies. The NOD/SCID mouse lacks T and B cells and therefore lack natural antibodies that could destroy infused human platelets [1]. Because of this property, human platelets given to the mouse intravenously circulate for several days, permitting the model to be used for testing the ability of human antibodies to cause platelet destruction *in vivo* [2–7]. Preliminary studies have demonstrated the usefulness of NOD/SCID mouse model to monitor the survival and immune destruction of human platelets. However, differences exist between the research groups regarding the

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Authors' contributions

T. Bakchoul designed the study, analyzed the data, critically wrote the manuscript and revised the intellectual content of the manuscript

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method of PLT injection, the amount and route of antibody injection and the preparation of blood samples collected from the animal making the results poorly comparable.

Basically, in all laboratories resting human platelets are injected intravenously into the mouse circulation, where they can, in the absence of platelet-reactive antibodies, circulate up to 48h [8]. After estimating a baseline value (100%) of human platelets, platelet-reactive antibodies (with or without drug administration) can be infused. The impact of these antibodies on the survival of human platelets can then be analyzed by taking blood samples over time from the mouse [8].

Methodological details that require attention in this model include: platelet preparation and resuspension in plasma or 'synthetic plasma', the concentrations and volume of applied analytes (platelet, antibody or drug), the route of platelet injection (retro-orbital injection or tail vein injection) and antibody injection (intravenous, intra-peritoneal). The method of data capture, including time points of blood sampling and subsequent sample preparation for analysis, percentage of circulating human platelets and software details should also be reported in detail. Additional steps required for answering the scientific questions, for example platelet preincubation with a drug of interest or an antibody in pooled plasma or 'synthetic plasma', should also be reported [2, 9]. Surprisingly, application procedures and the amount of injected platelets and antibodies have only been loosely defined and standardization should be carried out in order to improve the reproducibility of the procedures and to enable reliable comparison of the results.

This report is not didactic in relation to how to measure the survival of human platelets using the NOD/SCID mouse model. Its purpose is to suggest standardized procedures and define variables that should be considered when presenting methodology in published reports. The presented procedures were introduced and discussed during the meetings of the Subcommittee of Platelet Immunology of the Scientific and Standardization Committee Liverpool 2012 and Milwaukee 2014. Suggestions were introduced to the SSC members and the presented recommendations had unanimous agreement.

Adopting these recommendations will be of advantage for investigators and laboratories to reduce imprecision and harmonize results and will allow other laboratories to readily reproduce reported methods and findings and interpret results appropriately.

Recommendations

Platelet preparation from human blood

- It is recommended that blood should be taken under gentle suction (not vacuum suction) through a 21 G needle with a light tourniquet, or no tourniquet, with the donor in a sitting position. The first 2–3 mL of blood, which could potentially be contaminated by tissue factor present in skin or vascular cells, should be discarded.
- Blood should be collected from healthy donors of blood group 0 (no medication) into acid-citrated dextrose.

- Prostaglandine E1 (PGE1) should be added to blood samples at 50 ng/mL immediately after collection. Before any further manipulation, samples should rest for at least 10 min, but no longer than 60 min after collection to avoid activation.
- Platelet isolation should be performed using gentle protocols (first centrifugation 200×g, second one 700×g). Platelet pellets should be resuspended very gently after addition of PGE1 and adjusted to 2×10^9 /mL using platelet poor plasma from the same donor (or in citrated patient plasma for drug-induced thrombocytopenia cases).
- Adjusted platelets should rest for 30 min before injection.

Platelet injection into mice

- It is recommended to inject 200 μ L of platelet suspension (+PGE1).
- Platelet injection can be performed either via lateral tail vein injection (warming mice prior to the injection may help dilate the veins) or via retro-orbital injection (under general anesthesia).

Animals

- It is recommended to use Non-Obese Diabetic Severe Combined Immune Deficiency mice (NOD/SCID, Strain name: NOD.CB17-Prkdcscid/J, Stock number 001303; The Jackson Laboratory, Bar Harbor, Maine); age: 8–16 weeks, weight: 20–25 g. Mice should be age- and sex-matched in each experimental setting.
- It is our recommendation to include both genders and our experience indicates there are no differences between the genders for this protocol.
- For pilot studies at least three animals should be tested per experimental group. The number of animals should be determined based on power calculation if statistical analysis is needed to solidify the conclusion.

Blood sampling from mice

- It is recommended that 20–50 μ L blood is taken into 1 mL sample tubes containing 0.5 mL of a 1:9 mixture of 3.8% sodium citrate/Tyrode's-HEPES buffer and PGE1 at 50 ng/mL.
- It is recommended to collect blood samples by means of tail vein punctation prior to injection (t0) and 30min (t1), 1h (t2), 2h (t3), 5h (t4) and 24h (t5) after infusion of human platelets.
- Time points of blood sampling may be modified by the individual investigators depending on the nature of the experimental studies to better identify inter-group differences.

Platelet isolation from murine samples

- Density gradient centrifugation can be used to isolate the platelet fraction (human and murine) from murine whole blood samples, for instance with OptiPrep Density

Gradient Medium (Sigma-Aldrich). Platelets are thus found in the interface layer whilst other blood cells are pelleted.

Alternative method for platelet isolation from murine samples

- Platelet separation by density gradient centrifugation may, however, cause activation and loss of platelets, resulting in decreased recovery and bias in results obtained. An alternative is to fix blood samples, lyse the red blood cells and estimate the percentage of human platelets in the platelet gate (flow cytometer). For this method, reliable commercial kits have recently been launched (e.g. PerFix-nc Kit provided by Beckman Coulter). Advantages of this method include: no centrifugation steps necessary, all solutions needed for the experimental procedure are ready-to-use as provided with the kit, preparation time is reduced and fixation enables a measurement on the following day.

Timing of analysis

- It is recommended to analyze collected samples as soon as possible with unfixed cells and no later than 24h with fixed cells.

Analysis

Analysis should be performed by two color flow cytometry using a monoclonal antibody to identify the human platelets and another to identify the mouse platelets. The total platelet population (both mouse and human) can be gated by size and granularity. The fraction of human platelets in each sample is calculated as a ratio to mouse platelets. For calculation of survival curves, human platelets present in the baseline sample (30 min after injection) are considered to represent 100%.

Drug concentration

For many medications, the metabolism in the mouse is quite similar to humans. The model can therefore be used to identify metabolite-specific antibodies capable of causing thrombocytopenia in patients sensitive to certain medications (6). Drugs are injected intraperitoneally at doses to maximize the interaction with target cells and drug-dependent antibodies without becoming toxic to the animals. Sensitivity for antibody detection can be increased by injecting drugs/the drug at supra-pharmacologic (but sub-toxic) concentrations (6).

- Time of blood sampling should be based on the rate of formation of the drug metabolites or, if the literature information is insufficient to determine this, on the half-life of the parent drug.

Other factors

In order to maximize the antibody interaction with human platelets it is recommended to inject purified (Melon™ Gel, Pierce Biotechnology Inc.) IgG intravenously or, in the case of drug-dependent antibodies, to suspend human platelets in patient sample containing the drug-dependent antibodies prior to injection.

- IgG fractions should be freshly isolated (not stored at 4 °C) to avoid formation of IgG aggregates. Concentration of human IgG should be adjusted consistently in the test samples as well as in the negative control to a final concentration 5–10 mg/mL.
- In studies that are proposed to assess the ability of platelet-reactive antibodies to activate platelets, the activation status of human platelets should be assessed prior to the injection by analyzing the expression of P-selectin (after incubation with buffer and TRAP-6). These samples can be fixed and measured later together with the samples obtained to study human platelet survival.

Limitations and opportunities of the NOD/SCID mouse model

Although recent reports proved the usefulness of the NOD/SCID mouse model to study the role of HPA-1a antibodies in the clearance of antigen positive platelets in cases of PTP and NAIT, the role of drug-dependent antibodies specific for drugs and drug metabolites in drug-induced immune thrombocytopenia and ITP, other possible uses could be envisioned. These include studies of platelet storage conditions, assessment of platelet therapeutics, and studies of platelet destruction in heparin induced thrombocytopenia, thrombotic thrombocytopenic purpura or hemolytic uremic syndrome. One of the major limitations of this model is, however, the difficulty to study the function of human platelets due to the overwhelming number of mouse platelets in the system. Therefore this model is recommended for the study of human platelet survival but not for the assessment of human platelet function.

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