3. Development of Murine Model of Thrombocytopenia

3. DEVELOPMENT OF MURINE MODEL OF THROMBOCYTOPENIA.

3.1 INTRODUCTION

Use of animals in biological research and medicine has been in practice from the time chemical and biological molecules were discovered. The search of molecules useful to humans has been unending and the effects are initially studied in animals. Similar anatomy and physiology between human and animals, particularly the mammals have provoked the biological scientific community to assess and investigate the large range of mechanism and novel therapies in animal models before applying their research to or in humans. However the research on animals cannot be directly translated to humans as humans and other mammals are very complex organisms in which organs achieve distinct physiological functions in a highly integrated and regulated fashion (Barré-Sinoussi & Montagutelli, 2015). These involve a complex network of hormones, circulating factors and cells and cross-talk between cells in all the compartments. There are multiple levels of assessment of organisms by biologists, which include: molecules, cells, organs and physiological functions, in healthy or diseased conditions. The mechanism involved at all levels should be understood and described fully. With new technologies coming in most of the mechanisms involved can be studied using *in vitro* approaches (e.g., cell culture). These techniques have become very sophisticated to mimic the complex 3D structures of tissues (Barré-Sinoussi & Montagutelli, 2015). Some of these major scientific advances have replaced the use of animals. However, the exploration of physiological functions and systemic interactions between organs requires a whole organism and cannot be sufficed through the use of *in vitro* techniques. Hypotheses and models can emerge from *in-vitro* studies but they must be tested and validated in a whole organism,

otherwise they remain speculative. Scientists, as of today, are far from being able to predict the functioning of a complex organism from the study of separate cells, tissues and organs (Barré-Sinoussi & Montagutelli, 2015).

Development and assessment of novel vaccines and therapies are all dependent on Animal models and they are essential to address a variety of scientific questions in basic science. The use of animals is not only based on the vast similarities in the biology of most mammals with humans, but also on the fact that many human diseases often affect other animal species (Barré-Sinoussi & Montagutelli, 2015). These include not only infectious diseases but also some of common conditions such as Type I diabetes, hypertension, allergies, cancer, epilepsy, myopathies and so on. Not only are these diseases shared but the mechanisms underlying are often so similar that 90% of the veterinary drugs used to treat animals are identical or very similar to those used to treat humans (Barré-Sinoussi & Montagutelli, 2015) (ref). A number of major breakthroughs in basic science and medical research have been possible because of observations and testing on animal models. Most vaccines have been successfully developed using animal models. The Nobel Prize was awarded to Banting and McLeod in 1921, for establishing Type I diabetes treatment by insulin, in the dog (Best and Scott, 1923). Cellular therapies for tissue regeneration using stem cells have been engineered and tested in animals (Klug *et al.*, 1996). Many surgical techniques have been designed and improved in various animal species before being applied to humans. The discoveries in which animal models played a critical role are indeed numerous and led to many Nobel Prizes.

The usefulness and need of animal models to understand the etiology and treatment of human illnesses has become overwhelmingly clear (Semple, 2010). Model systems generally provide a convenient system for studies for evaluation of potential therapeutic modalities. Considering the difficulties associated with evaluating Thrombocytopenia treatments in human, it may be advantageous to assess new therapies in animal models of the disease to allow systematic and quantitative investigation. Suitable animal models should provide a reproducible condition of severe thrombocytopenia, mediated by anti-platelet antibodies or Drugs. Ideally, the model should allow accurate and precise quantification of Thrombocytopenia; such a model may facilitate mechanistic evaluation of therapy (Hansen & Balthasar, 2001). Among the several animal models that were examined, for drug-induced thrombocytopenia, some were passive thrombocytopenic kind while others had their own unique nature. For example, in 1964, Wakisaka *et al.* studied the effects of quinidine on immune thrombocytopenia in dogs. Subsequently, Kekomäki *et al.* (1977) demonstrated the thrombocytopenic effects of immune complexes in rabbits immunized with aspirin.

There are very few drugs that are prescribed for decreasing the blood platelet count. Antiplatelet medications used to treat or prevent thrombosis in a variety of cardiovascular disorders include acetylsalicylic acid (aspirin), P2Y12 receptor antagonists (clopidogrel, ticlopidine, prasurgrel, cangrelor, tocagrelor), phosphodiesterase inhibitors (dipyridamole, anagrelide, cilotazol), and GPIIb/IIIa inhibitors (abciximab, eptifiibatide, tirofiban, lamifiban).

Anagrelide: Anagrelide is an imidazo (2-1-b) quinazolin-2-1 compound that was originally developed as an antithrombotic agent because of its powerful anti aggregating

effect on platelets (Fleming & Buyniski, 1979). It is now an established drug for treatment of thrombocytosis and certain myeloproliferative disorders (Balduini *et al.*, 1992; Silverstein *et al.*, 1988). It inhibits cyclic nucleotide phosphodiesterase and the release of arachidonic acid from phospholipase, possibly by inhibiting phospholipase A2. The specific mechanism by which anagrelide induces thrombocytopenia remains unclear. In one of the clinical study published by Silverstein and coworkers, it was shown that anagrelide does not alter the cellularity or the number of megakaryocytes in the bone marrow, or the survival of circulating platelets (Silverstein *et al.*, 1988). Thereby it does not inhibit the proliferation of megakaryocytic-committed progenitor cells (colony-forming units) in vivo. However, suprapharmacologic concentrations may inhibit megakaryocyte colony expansion in cell culture systems. Therefore, anagrelide-induced thrombocytopenia does not arise as the result of direct stem cell toxicity or direct inhibition of megakaryocytopoiesis (Mazur *et al.*, 1992). Anagrelide is licensed for use in the United States and Europe for the treatment of patients with thrombocythemia associated with all myeloproliferative disorders. The broadest experience with anagrelide has been generated by the multicenter phase 2 clinical trial conducted by the Anagrelide Study Group ("Anagrelide, a therapy for thrombocythemic states: experience in 577 patients. Anagrelide Study Group," 1992). Among 577 patients with primary thrombocythemia (median platelet count, 990,000/µL), 94% response rate (defined as a reduction in platelet count by 50% or maintenance at levels of <600,000/µL for at least 4 weeks) was reported when treated with anagrelide.

HYDROXYUREA: Hydroxyurea is a hydroxylated analogue of urea and an anti-metabolite which inhibits the enzyme ribonucleotide reductase that is necessary for DNA

synthesis and cell cycle replication (Platt, 2008). It reduces intracellular deoxynucleotide triphosphate pools and acts as an S-phase-specific agent with inhibits DNA synthesis and leads to cellular cytotoxicity. It however does not interfere with RNA or protein synthesis. It is mainly used in the treatment of solid tumors and myeloproliferative diseases, the latter because of its effects in reducing excessive production of red blood cells (polycythemia vera), white blood cells (chronic myelogenous leukemia) or platelets (essential thrombocythemia). Hydroxyurea also increases the production of fetal hemoglobin via an unknown mechanism of action, thereby increasing overall hemoglobin levels and decreasing sickling in patients with sickle cell anemia. Hydroxyurea was first approved for use in the United States in 1967 as an antineoplastic agent for therapy of melanoma, chronic myelogenous leukemia, ovarian carcinoma, and head and neck cancers. It is also utilized by many physicians for the initial treatment of essential thrombocythemia wherein platelet count goes very high. Although the drug has a broad dose–response range, mild side effects and theoretically some mutagenic risk, quickly reverses on discontinuation of the drug without any unwanted myelosuppression. Cortelazzo *et al.* (1995) reported decrease in the platelet count to below 600,000 per cubic millimeter, in patients treated with hydroxyurea .

Aspirin: Aspirin, also known as acetylsalicylic acid, is a medication used to treat pain, fever, and inflammation. It decreases platelets by inhibiting platelet prostaglandin synthesis as well as ADP- and collagen-induced platelet release reaction. The mechanism of inhibition is not known but may involve protein acetylation as aspirin is known to acetylate platelet proteins related to cyclo-oxygenase, the prostaglandin G2 biosynthetic enzyme group (Roth & Majerus, 1975).

Vancomycin is an antibiotic used to treat a number of bacterial infections (Liu *et al.*, 2011). Skin infections, bloodstream infections, endocarditis, bone and joint infections, and meningitis caused by methicillin-resistant *S. aureus* could be treated by Vancomycin given intravenously (Liu *et al.*, 2011). Vancomycin was first isolated in 1953 by Edmund Kornfeld from a soil sample collected from the interior jungles of Borneo by a missionary. The organism that produced it was eventually named *Amycolatopsis orientalis* (Levine, 2006). Vancomycin was first sold in 1954. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system (WHO, 2016). There have been many studies which suggest role of vancomycin in lowering blood platelet count in patients. In a study, vancomycin-dependent antiplatelet antibodies were detected by (Von Drygalski *et al.*, 2007), in patients receiving the antibiotic, leading to thrombocytopenia.

In our present study, we used Vancomycin, Aspirin, Hydroxyurea, Cyclophosphomide and anagrelide to make drug induced model of thrombocytopenia. Use of antiplatlet antibodies was also included to mimic the immune thrombocytopenia condition.

3.2 MATERIALS AND METHODOLOGY:

Experimental models:

Rodents, rat and mice were used to create drug-induced Thrombocytopenia and immune Thrombocytopenia respectively.

Four month old female albino rats (*Rattus rattus norvegicus*) of Wistar strain weighing 200 – 225 g were used for various drug induced Thrombocytopenia.

Eight weeks Balb/C mice weighing approximately 30gms were used to induce immune Thrombocytopenia.

Experimental Design:

Drugs / Compound used for induction of thrombocytopenia

- **Vancomycin** (5mg/kg BW and 10mg/kg BW, Intravenous)
- **Hydroxyurea:** (15 mg/Kg BW and 25 mg/Kg BW, Oral intubation).
- **Hydroxyurea + Aspirin :** (15mg+12.5 mg/Kg BW and 25 mg + 15 mg/Kg BW, Oral intubation)
- **Anagrelide (genric: Xagrid 0.5mg capsule):** (10µg/kg BW and 25 µg/Kg BW, Oral Intubation)
- **Antiplatelet Antibody (CD41, Cat No. Biolegend, USA)**

Investigation for the required drug was done according to CPCSEA guidelines (827/ac/04/CPCSE A). Each drug was administered to 6 animals of similar weight, age and sex. The dose and route of administration were as stated above. Animals were treated with respective drugs for the period of 4 weeks. Blood test for Hematology count was

done once every week and animals were observed for any abnormal behavioral changes throughout the experimental period.

Parameters evaluated:

Animals were bled under mild anesthesia through retro-orbital puncture. 200- 300µl of anticoagulated blood was used for the blood cell count. Blood counts were evaluated on Mindray 2300 auto hematoanalyser. Platelet count of <150,000/µl was considered thrombocytopenic. Other parameters such as total WBC count, Total RBC count and Hb were recorded and evaluated for the assessment of health of animals. Blood parameters were checked for each group before dose regime was started to ensure that there is no ongoing infection in the animal.

Flow Cytometrey Analysis:

Materials:

1. (+) Biotin N-hydroxysuccinimide ester (NHS-biotin) (Sigma Aldrich).
2. Dimethyl sulfoxide (DMSO) (SRL Chemicals, Gujarat).
3. Saline for injection of NHS-biotin: 0.9% NaCl, unbuffered. (Clarius Pharma, Gujarat, INDIA)
4. Antibodies:
    a) LEAF™ Purified anti-mouse CD41 Antibody clone MWReg30 (BioLegend Cat. No. 133910, CA, USA),
    b) PE anti-mouse CD41 Antibody conjugated to phycoerythrin (PE) Antibody clone MWReg30 (BioLegend Cat. No. 133905, CA, USA)
5. Streptavidin conjugated to allophycocyanin (APC) (BioLegend Cat. No.405207, CA, USA).

6. Aster Jandl citrate-based anticoagulant (AJ): 85mM sodium citrate dihydrate, 69mM citric acid, 20mg/ml glucose, pH 4.6.
7. Platelet wash buffer: 140mM NaCl, 5mM KCl, 12mM sodium citrate, 10mM glucose, 12.5mM sucrose, pH 6.0.
8. Platelet buffer: 0mM HEPES, 140mM NaCl, 3mM KCl, 0.5mM $MgCl_2$ hexahydrate, 0.5 mM $NaHCO_3$, 10mM glucose, pH 7.4. Titrated with NaOH.
9. Needles 25 gauge 5/8 in., syringes 1ml. (BD Biosciences)
10. Warm water (60^{o} C).
11. Alcohol Swabs
12. Flow cytometer (BD FACS Aria III, BD Biosciences).

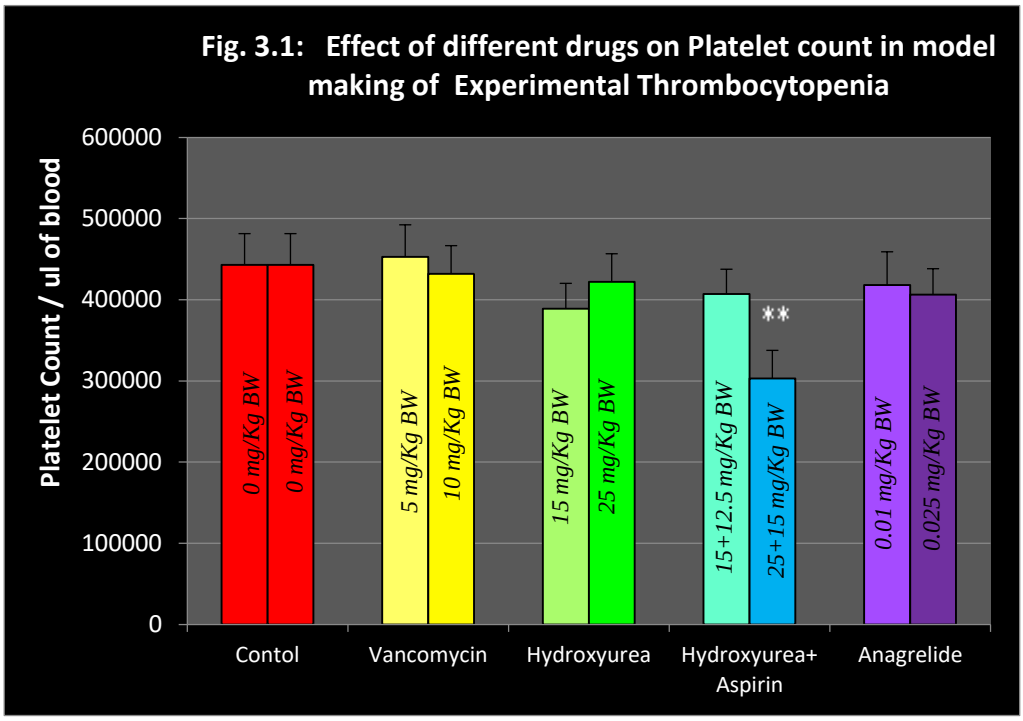
Experimental Setup:

- Four Balb/C mice regardless of sex were selected for the study. The animals were acclimatized and were given free access to food and water for the entire period of experiment.
- Animals were injected with NHS Biotin **600µg/ mice**.
- After 2 hrs of injection animals were injected with Antiplatelet antibody **4µg/ mice** .
- The mice were bled at 0 hrs (before antibody injection).

  6 hrs after antibody injection.

  24 hrs after antibody injection.
- The Blood was obtained in Aster Jandl citrate-based anticoagulant and was utilized to obtain Platelet Rich Plasma (PRP). PRP obtained was stained with Streptavidin conjugated to APC and PE Conjugated CD41 antibody.
- Platelet population was selected by FSC SSC gating. CD41 positive population was screen for Streptavidin positive cells.

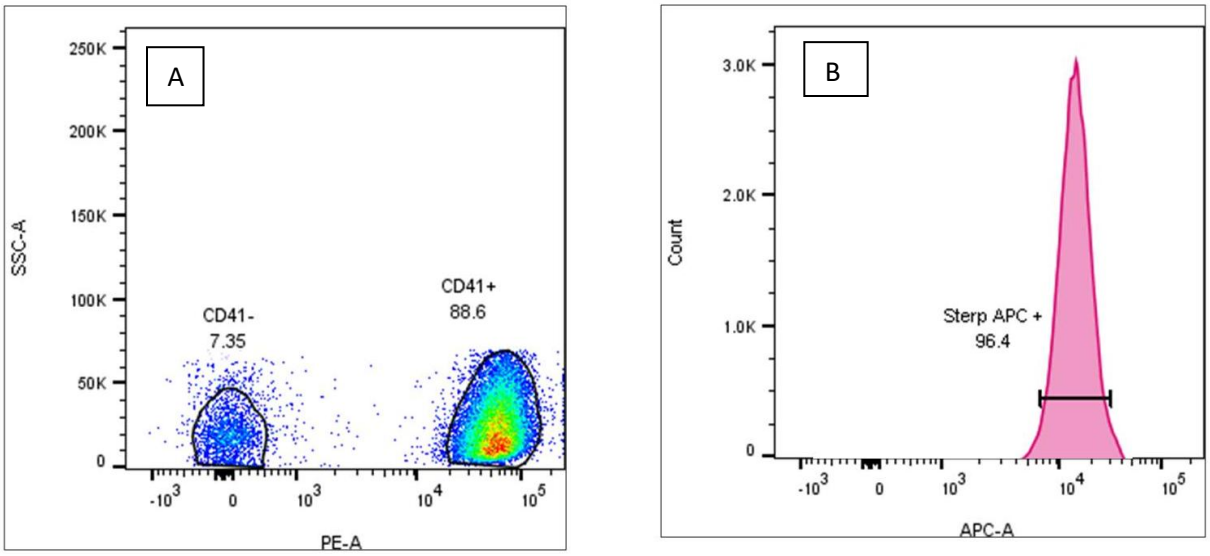
3.3 RESULTS



Results are expressed as mean ± S.E. for n=6. *p<0.05, ** p<0.01, *** p<0.001 Control is compared treatment.

Results: Vancomycin *i.v.* did not show any change in blood platelet count (p>0.05). Animals administered with Anagrelide a prescribed molecule for decreasing blood platelet count in human subjects, did not decrease murine platelets to significant levels (p>0.05), at the same time other hematological parameters were also normal. Hydroxyrurea alone at dose of 25 mg/kg B.W. did not decrease the blood platelet count, however, a combination of Hydroxyurea with Aspirin 25 + 15 mg/kg B.W. decreased the blood platelet count to 2,00,000 cells/µl blood (p<0.05). (Fig. 3.1)

Fig. 3.2 *In vivo* Biotinylation of Platelets for immune thrombocytopenia model



A: Represents CD41 positive and CD41 negative populations from PRP. 2 hr post Biotin injection. CD41 positive cell population was further selected to identify the sub-population of Streptavidin positive platelets.
B: Represents the population of Streptavidin positive platelets 2 hr post induction

Mice were injected with NHS-biotin which labels all the platelets in the circulation 2hrs post biotin injection in tail vein. Blood was collected at regular intervals to identify the window where platelets are affected the most. The results obtained suggest that there are an adequate number of platelets (FIG 3.2 A) in the circulation and over 95% of blood platelets were labeled by Streptavidin within 2 hrs of injection (FIG 3.2 B).

Fig.3.3 *In vivo* Biotinylation of Platelets for immune Thrombocytopenia model 6h antibody post induction

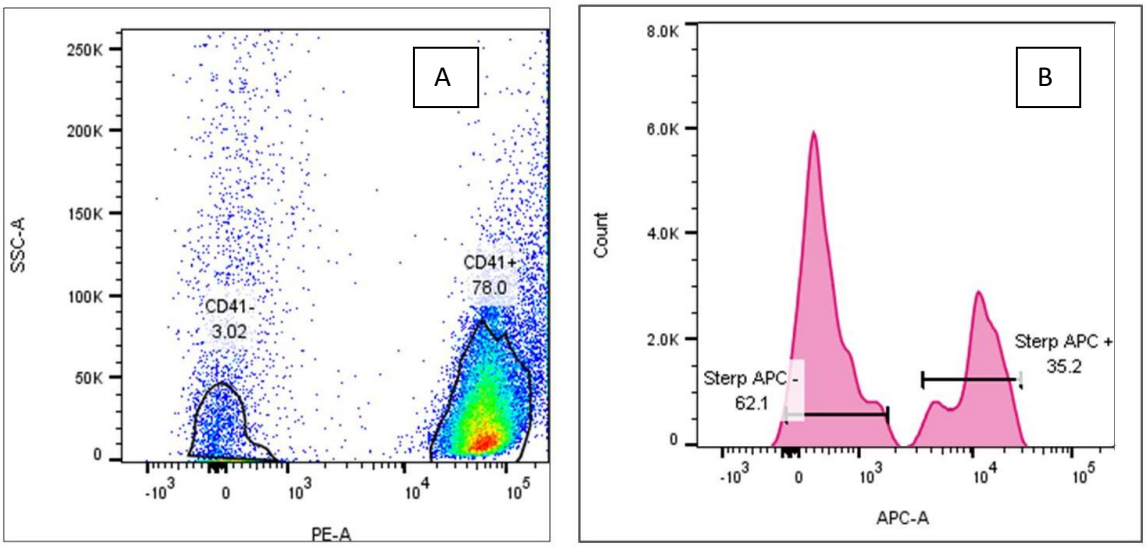


FIG A: Represents CD41 positive and CD41 negative populations from PRP. 6 hr post antibody induction. CD41 positive cell population was further selected to identify the sub-population of Streptavidin positive platelets.
FIG B: Represents the population of Streptavidin positive platelets 6 hr post induction with antibody.

Six hours after Antiplatelet antibody injection, nearly 50% of the platelet population in the circulation were removed (FIG 3.3 B) and new platelets had taken over (Population positive for CD41, but negative for Streptavidin) while the older platelets are present in the circulation are positive for both CD41 and Streptavidin.(Fig3.3 A & B).

Fig 3.4: *In vivo* Biotinylation of Platelets for immune Thrombocytopenia model 12 Hrs antibody post induction

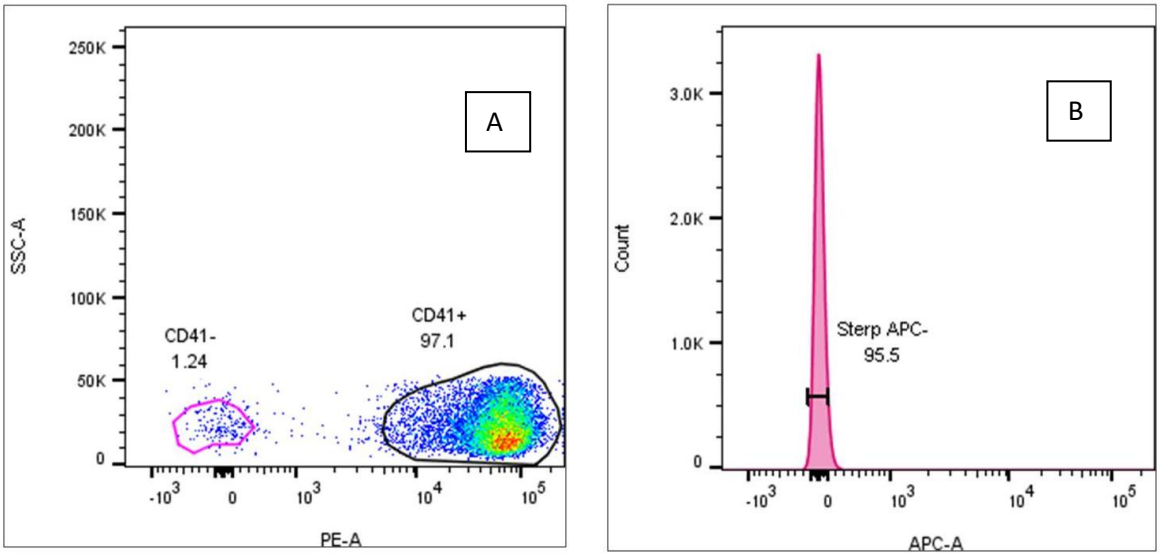


FIG A: Represents CD41 positive and CD41 negative populations from PRP. 12 hr post antibody induction. CD41 positive cell population **(Black line)** was further selected to identify the sub-population of Streptavidin positive platelets.
FIG B: Represents the population of Streptavidin positive platelets 12 hr post induction with antibody.

12 hrs post Antiplatelet antibody induction, nearly all the antibody bound platelets were removed from the circulation (Fig 3.4B). However, due to lower platelet count innate mechanism of platelet production and Megakaryopoesis new platelets were put into circulation to maintain the threshold of platelets which can be substantiated by Fig. 3.4A.

3.4 DISCUSSION:

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by the production of autoreactive antibodies against one's own platelets, resulting in increased platelet destruction by phagocytic macrophages in the reticuloendothelial system (Cines & Blanchette, 2002; Fabris *et al.*, 2004; Hou *et al.*, 1995; Michel, 2009; S. Mehta, 2000; Stasi *et al.*, 2008; Zhou *et al.*, 2005).

The etilogy of the disease remains mostly unclear and thereby it can be fatal in some cases. Due to the difficulties associated with evaluating Thrombocytopenia treatments in human, it is a good practice to assess new therapies in animal models of the disease to allow systematic and quantitative investigation. Suitable animal models should provide a reproducible condition of severe thrombocytopenia, mediated by anti-platelet antibodies or Drugs. Vancomycin, a 4th generation antibiotic is used in various diseases; however it is associated with thrombocytopenia in many patients (Kuruppu *et al.*, 1999; Marraffa *et al.*, 2003; Von Drygalski *et al.*, 2007). It stimulates the formation of platelet-reactive antibodies of the IgG class and the IgM class in the blood and thereby causes severe thrombocytopenia in humans (Christie *et al.*, 1990). We therefore used Vancomycin to simulate thrombocytopenia in murine model. However, we failed to observe any significant drop in platelet count with respect to untreated (Control) animals in both low (5mg/Kg BW) and High (10mg/ Kg BW) dose of Vancomycin. Similarly, we used Hydroxyurea, a known drug used in acute myeloid cancer and Essential thrombocytemia. This drug has a broad dose–response range, mild side effects, and theoretically little mutagenic risk (Carobbio *et al.*, 2010; Cortelazzo *et al.*, 1995). Discontinuation of the drug is known to quickly reverse any unwanted myelosuppression. Murine model, in the present study, did not respond to Hydroxyurea treatment at both the

doses (15mg/Kg & 25mg/Kg). However, Hydroxyurea (25 mg/kg BW), when administered along with Aspirin (15 mg/Kg), did result in a moderate non significant decrease in blood platelet. According to National Heart Lung and Blood Institute, platelets less than 150,000/µl of blood, is clinically assigned as thrombocytopenia. Anagrelide a new generation drug of essential thrombocythemia also did not show any effect on murine blood platelet count in low (10µg/Kg) as well as high (25µg/kg) doses. Lane *et al.* (2001), administered metanagrelide, an anagrelide metabolite produced in the liver of humans, to mice which significantly reduced the blood platelet count. In our studies the angrelide treatment did not show any decrease in blood platelet count as compared to control, probably because rat liver was incapable of metabolizing anagrelide.

The present study is the first attempt to induce thrombocytopenia using pharmacological drugs, Vancomycine, Hydroxyurea and combination of Hydroxyurea + Aspirin in the above mentioned concentrations in Murine model. Unfortunately, we did not succeed in developing a clinically relevant Thrombocytopenic murine model. Thereby we used antibodies to induce thrombocytopenia in Balb/c mice as per Marjon *et al.* (2009) The significant reduction in blood platelet count was recorded by Flowcytometry. After 6hrs of antibody treatment there was considerable (> 50%) decrease in platelet count while 12hrs after antibody treatment all the platelets were replaced by new platelets (although less) through megakaryopoiesis (confirmed by no peak of streptavidin positive cells).

The present study ruled out the use of rat as a thrombocytopenic model for further study. We therefore performed our next experiment using throbocytopenia mice model induced by CD41 antibody.

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