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Original Article

Evaluation of Immunosuppressive Effects of Azathioprine and Cyclophosphamide in CD1® Mice by Flow Cytometer

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ABSTRACT

Introduction: Qualitative and quantitative analysis of single cells treated with immunomodulators can be done by FACS. T cells, B cells and NK cells, designated as CD cells, are the immunological biomarkers of various haematological conditions which are attributed to prognosis and diagnosis of related disorders. Azathioprine and Cyclophosphamide are immunosuppressive agents, having different mode of action and classification which are been used clinically. Here, we have compared the dose of Azathioprine and Cyclophosphamide in CD-1® mice through lymhocytes' enumeration by FACS to compare the potency of immunosuppression. **Methods:** Randomly divided male and female mice were treated with AZA 20, AZA 50 and CPM 20 for four days, Body weight and feed consumption was taken on day 1 and 5. Collected blood samples were mixed with antibodies. Samples were lysed by lysing solution. Repeated washing and centrifugation yields leucocytes in each tube for estimation. Lymhocytes' enumeration was performed for CD3, CD4, CD7, CD8, CD16, CD19 and CD45 and percent population of CD markers were evaluated. **Results and Conclusion:** All the animals were normal throughout treatment. No significant change in body weight, percent body weight change and food consumption was observed. No gross lesions were observed. Total lymhocytes' counts were decreased. Double negative tregs of $CD4^-$ and $CD8^-$ in female mice showed potent immunosuppressive effects and sensitivity towards female gender. Azathioprine was less immunosuppressive than Cyclophosphamide at 20 mg/ kg body weight.

Key words: CD cells, Leucocytes, Fluorescent Activated Cell Sorting System (FACS), Lymphocyte enumeration, Immunotoxicity.

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INTRODUCTION

FACS offers new approaches to study various immunomodulators and their effects. The technique measures the characteristics of single cells, determined by visible and fluorescent light emissions from the markers on the cells. As the liquid flow moves the suspended, labeled cells past a laser that emits light at a particular wavelength, the specific markers attached to the cell fluorescent. The fluorescence emission from each cell is collected, and subsequent electrical events are analyzed on a computer that assigns a fluorescence intensity value to each signal in FCS data files. Each FCS data file thus consists of multi-parametric descriptions of thousands to millions of individual cells.[1]

Toxicological studies include clinical observation, clinical pathology/ chemistry end point measurement and macroscopic/microscopic pathologic examination. This technique is advantageous over slide scoring method of various tissues and blood where we study the various biomarkers for immune related disorder at a single time in FACS. Flow cytometers are designed to enumerate the absolute numbers and percentages of lymphocyte populations, such as subsets of T cells, B cells, and NK cells. The major subgroups of lymphocytes, namely T, B, and NK cells, can be distinguished by surface markers referred as CD markers by FACS. The relative proportions of these subpopulations can change in various clinical situations, such as innate or acquired immune deficiencies.[2,3]

Azathioprine is a purine analogue, inhibiting DNA replication in all rapidly dividing cells, including lymphocytes. Cyclophosphamide is an alkylating agent related to the nitrogen mustards. It is powerful immunosuppressant acting as it cross-links DNA in actively multiplying cells. It is strongly immunosuppressive, as it inhibits normal primary and secondary immune responses.[4]

The objective of this research was to compare the immunosuppressive effects of Azathioprine and Cyclophosphamide at different dose levels by FACS through lymhocytes' enumeration.

MATERIALS AND METHOD

Materials

Chemcials

Chemical Name (1)	:	Cyclophosphamide Monohydrate
Lot N°	:	120M1253V
Manufactured by	:	Sigma-Aldrich, 12, Bommasandra-Jigani Link Road, Bangalore
Chemical Name (2)	:	Azathioprine
Lot N°	:	SLBD 7131V
Analysed Purity	:	> 97%
Manufactured by	:	Sigma-Aldrich, 12, Bommasandra-Jigni Link Road, Bangalore

Instruments and Equipment

Tattoo Machine	:	Animal Identification & Marking System, Inc.
Balances	:	1. Sartorius AG, TE 2101 (Capacity–0.1 g to 2100 g)
		2. Adair Dutt, Semi-micro Electronic

		(Capacity–10 mg to 200 g) 3. Sartorius AG, BT1245 (Capacity–1 mg to 120 g)
Flow Cytometer	:	BD FACSVerse TM, Becton Dickinson company.
Refrigerators	:	Godrej & Boyce, LG Electronics
Cooling Centrifuge	:	Remi Instruments
Magnetic Stirrer	:	DBK Interlink

Antibodies

CD3e-PerCP-Cy™5.5 Hamster Anti-Mouse CD3e (BD Pharmingen™)

CD4-FITC Rat Anti-Mouse CD4(BD Pharmingen™)

CD8-APC Rat Anti-Mouse CD8a (BD Pharmingen™)

CD 16-PE Rat Anti-Mouse CD16/CD32 (BD Pharmingen™)

CD 19-APC Rat Anti-Mouse CD19 (BD Pharmingen™)

CD 45-PerCP Rat Anti-Mouse CD45 (BD Pharmingen™)

CD7-nk cells-PE Rat Anti-Mouse NK-T/NK Cell Antigen (BD Pharmingen™)

Method

Healthy young CD-1® (RccHan: Wist) mice were obtained from the Animal Breeding Facility, Jai Research Foundation. At the initiation of the dosing period, rats were 9 weeks old. Female rats were nulliparous and non-pregnant. Mice is the preferred test system because it is a readily available laboratory model, and has been shown historically to be an acceptable model for immunotoxicity testing and is recommended by the EPA, OECD and other regulatory authorities.

This research was carried out in compliance with the 'Guidelines for Laboratory Animals Facility' issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The experimental animals were fed with Teklad global 16% protein rodent diet, obtained Harlan Laboratories, *ad libitum*. The animals were provided potable water *ad libitum* (Reverse Osmosis water filtration system) in polypropylene bottles.

Animals were weighed on the day of randomization. Body weight of all the animals were within ± 20% of mean body weight of each sex on the day of dosing.

After grouping, each animal was marked with permanent animal number. Individual mice were identified with unique numbers tattooed on the tail using a tattoo machine.

Total 20 male and 20 female CD-1® mice were randomly divided into four groups: G1 (Vehicle Control), G2 (Cyclophosphamide monohydrate: 20 mg/kg b. wt./day), G3 (Azathioprine: 20 mg/kg b. wt./day) and G4: (Azathioprine: 50 mg/kg b. wt./day)A concurrent vehicle control group of rats received vehicle reverse osmosis water only. All animals were observed twice in a day for clinical signs and for mortality and morbidity. Body weight of individual animal was determined on day of dosing and on terminal sacrifice. Feed consumption of individual male and female animals was determined during treatment period. At terminal sacrifice, blood was collected from each animal for enumeration of lymphocytes.

At scheduled sacrifice, male and female mice were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination. Blood samples were analyzed using FACSVerse™ flow cytometer for evaluation of percent population of NK T/B cell surface markers.

For the labeling of blood samples with antibodies, 100 µL of blood was taken in vial. Different antibodies viz., CD3, CD4, CD7, CD8, CD16, CD19 and CD45 were added to each blood sample. After adding the antibodies, content of vials was mixed gently. Vials were incubated at room temperature for 13-15 min. in dark. 2 mL of 1 x lysing solution was added in each vial and incubated at room temperature for 13-15 min in dark. Then centrifuged at 1700-2000 rpm for 5-6 min. 2 mL of sheath fluid was added. Then centrifuge at 1700-2000 rpm for 5-6 min again. Supernatant of each vial was discarded and 500 µL of sheath fluid was added. Then all the samples were run in BD FACSVerse™ system.[5]

RESULTS

No any gross lesions were observed in macroscopic evaluation of animals. No clinical sign and mortality was observed in animals. No difference in body weight, % body weight change and food consumption was observed in all treatment groups when compared to vehicle control group.

Similar lymphocytes' suppression was observed in CPM 20 and AZA 50 treated animals in male and female mice when compared to control group. Similar suppression of $CD19^{-}CD16^{+}$ tregs was observed in CPM 20 and AZA 50 in both male and female mice when compared to control group. Similar suppression of $CD45^{+}CD16^{+}$ tregs was observed in CPM 20 and AZA 50 in both male and female mice when compared to control group. Similar suppression of $CD19^{+}CD45^{+}$ tregs was observed in CPM 20 and AZA 50 in both male and female mice when compared to control group. Similar suppression of$CD3^{+}CD7^{+}$ and $CD3^{-}CD7^{+}$ tregs was observed in CPM 20 and AZA 50 treated groups in both male and female when compared to control groups. Similar suppression of $CD8^{-}CD3^{-}$ and $CD8^{-}CD4^{-}$ tregs was observed in CPM 20 and AZA 50 treated groups in female when compared to control group.

DISCUSSION

Immunofluorescence analysis by flow cytometry is used widely as part of the laboratory determination of blood subset counts. These measurements have other clinical applications, including transplantation monitoring and diagnosis of immunodeficiencies both primary and secondary.[6,7]

Lymphocytes and other leukocytes express different surface molecules which can be used for distinguishing cell population. A systemic nomenclature called CD system has been developed, in which the term CD refers to cluster of designation. These different cell surface molecules are detected with specific monoclonal antibodies, each of these molecules are given CD number. T cells can be subdivided into two distinct non-overlapping populations: a subset which carries the CD4 marker (MHC class II receptor) and mainly 'help' or 'induce' immune responses, and the other subset which express the CD8 marker (MHC class I receptor) and it is predominantly cytotoxic. B lymphocytes represent about 5-15% of the circulating lymphoid pool, and are classically defined by the presence of surface immunoglobulins. These immunoglobulin markers are made by the B cells themselves, and are inserted into the surface membrane where they act as specific antigen receptors.[8,9]

CD45 (lymphocyte common antigen) is a receptor-linked protein tyrosine phosphatase that is expressed on all leucocytes, and which plays a crucial role in the function of these cells and T cell activation.[10] Engagement of cell surface receptors like CD19 by monoclonal antibodies can have

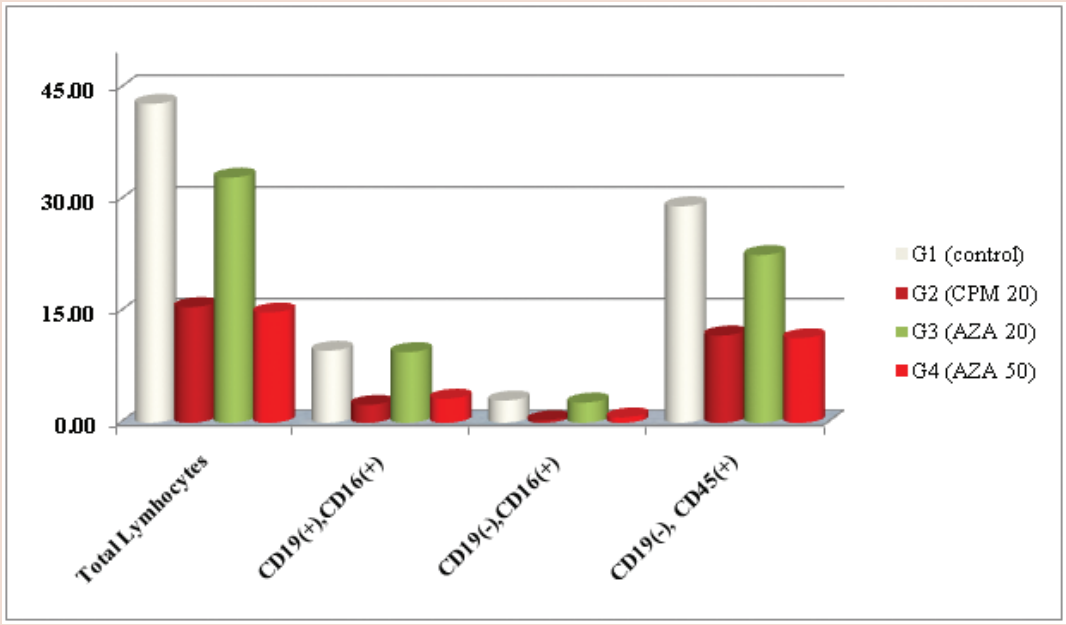

Figure 1: Graph of lymphocytes and CD cells (CD16, CD19 and CD45) Vs. % cell count for male mice.

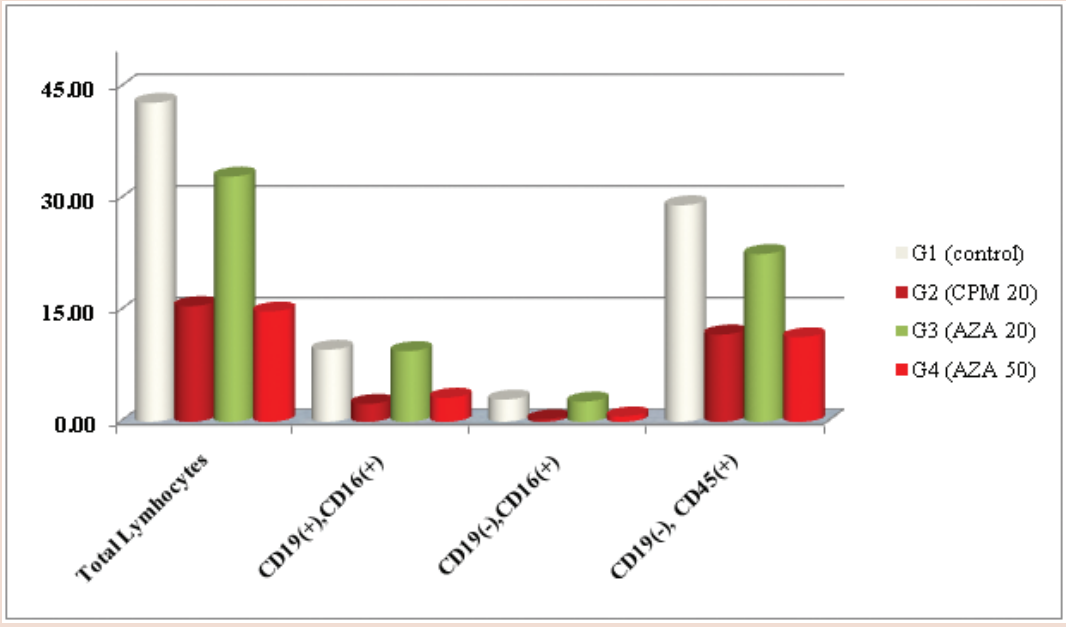

Figure 2: Graph of lymphocytes and CD cells (CD16, CD19 and CD45) Vs. % cell count for female mice.

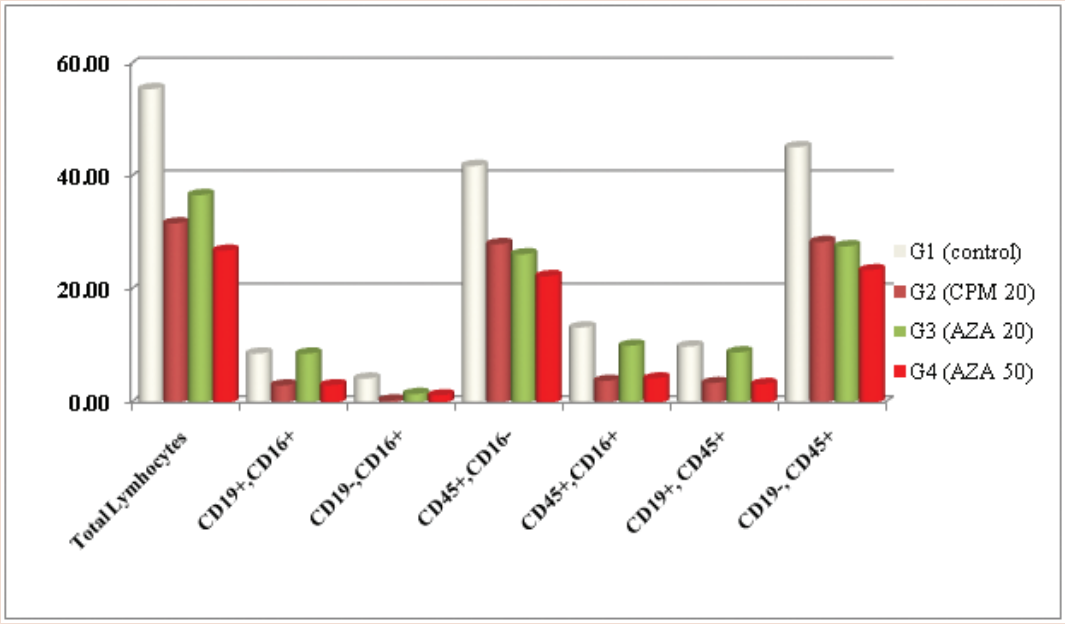

Figure 3: Graph of lymphocytes and CD cells (CD3, CD7-nk cells) Vs. % cell count for male mice.

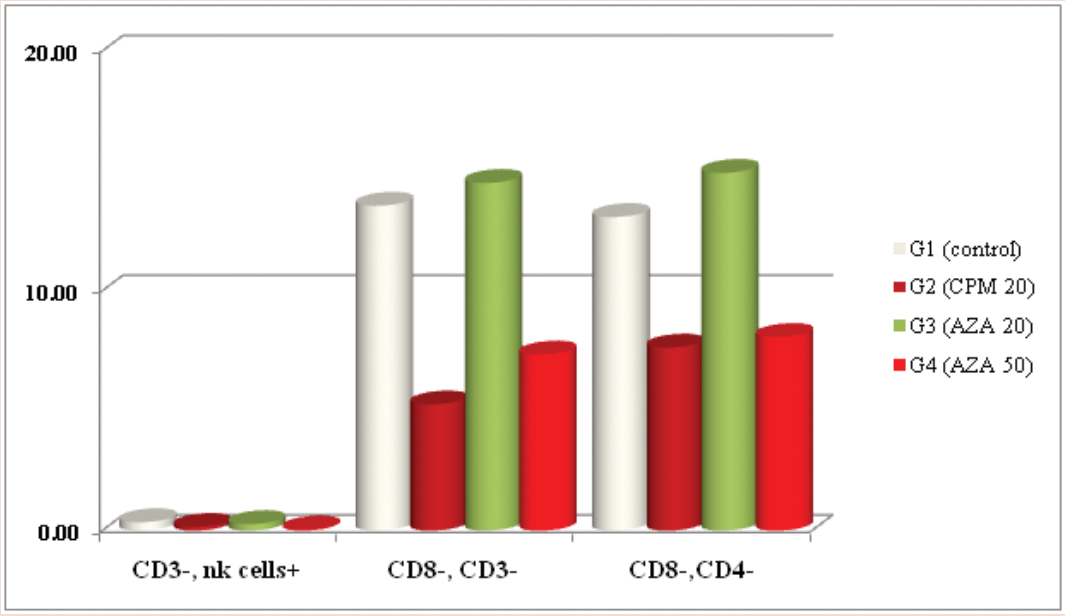

Figure 4: Graph of lymphocytes and CD cells (CD3, CD4,CD7-nk cells,CD8) Vs. % cell count for female mice.

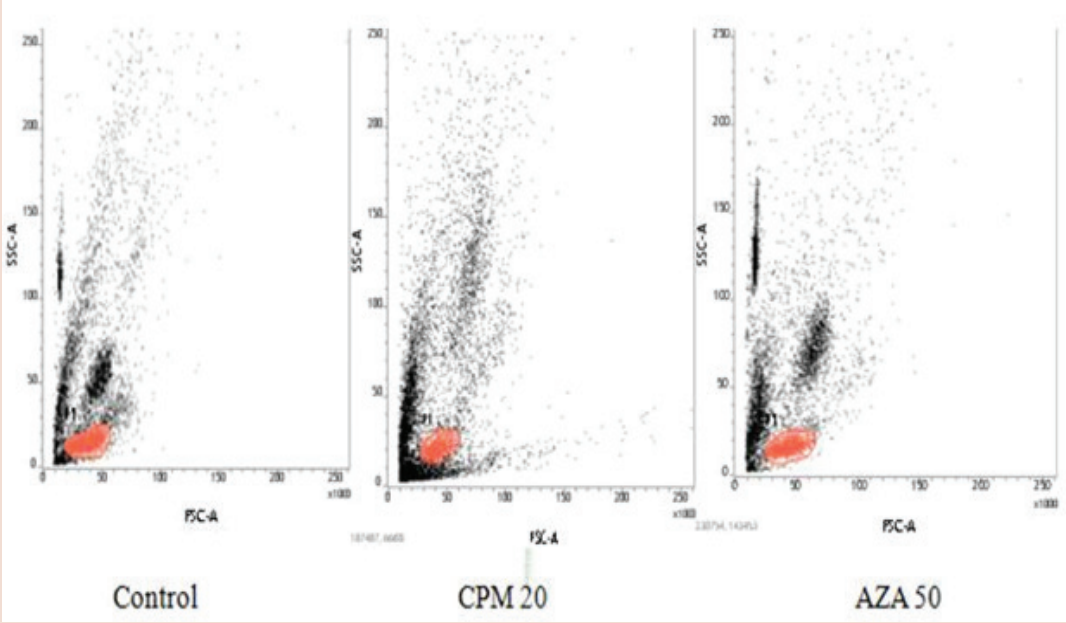

Figure 5: Facs parent plots of lymphocytes for control, CPM 20 and AZA 50.

anti-tumor effects by the activation of signal transduction pathways which control cell cycle progression and programmed cell death.[11]

Natural killer cell, referred to as large granular lymphocytes because of their appearance, represents about 15% of blood lymphocytes and expresses neither T protein couple receptor nor B protein couple receptor antigen receptors.[8] Natural killer cells were originally described for their ability to kill target cells without prior stimulation. These cells, which express the CD16 marker, are involved in antibody dependent cellular cytotoxicity.[12]

Cyclophosphamide is a potent immunosuppressive agent, capable of inhibiting both humoral and cell-mediated immune responses.[13] It is a cytotoxic, myelosuppressive alkylating agent that cross-links DNA helixes to prevent their separation, thus preventing the formation of a DNA template.[14,15] It suppresses both cell-mediated and humoral immunity, and it may suppress mononuclear phagocytic function. However, cyclophosphamide principally targets lymphocyte proliferation.[16]

Azathioprine interferes with T-lymphocyte function more than B-lymphocyte function, inhibiting cell-mediated immunity and T-lymphocyte dependent antibody synthesis. Azathioprine is less immunosuppressive than cyclophosphamide, but it is usually better tolerated than cyclophosphamide.[4]

Azathioprine is a cytotoxic antimetabolite that is converted to 6-mercaptopurine in the liver. It is a purine analogue that functions as a competitive purine antagonist, thereby inhibiting cellular proliferation. Azathioprine is less toxic to resting cells than cyclophosphamide and therefore has fewer side effects.

It also suppresses macrophage function, which reduces inflammatory cytokine production and phagocytic efficiency.[4,14,17,18]

CONCLUSION

In this experiment, Cyclophophamide at 20 and Azathioprine at 50 mg/kg body weight have similar immunosuppressive effects on T cells ($CD3^+$, CD4, CD8), B cells (CD19, CD45) and natural killer cells ($CD3^-$, CD16) population in male and female mice.

Female mice could be considered more sensitive than male as both helper and cytotoxic T cells ($CD4^-$ and $CD8^-$) suppressed by Cyclophophamide at 20 and Azathioprine at 50 mg/ kg body weight. This research results can be helpful for comparison in future studies where lymphocytic enumeration is a part of experiment.

ABBREVIATION USED

AZA 20: Azathioprine treatment at 20 mg/kg body weight; **AZA 50:** Azathioprine treatment at 50 mg/kg body weight; **B cells:** B lymphocytes; **CD cells:** Clusters of Differentiation cells; **CPM 20:** Cyclophosphamide at 20 mg/kg body weight; **EPA:** Environmental Protection Agency; **FACS:** Fluorescence Activated Cell Sorting System; **MHC:** Major Histocompatibility Complex; **NK or nk cell:** Natural Killer Cells; **OECD:** Organisation of Economic Co-operation and Development; **T cells:** T lymphocytes.

IMPORTANCE OF THE PAPER

Azathioprine and Cyclophosphamide are the immunosuppressive agents which have different mode of actions and used clinically. Here we validated the lymphocyte enumeration technique through flow cytometer and conclude identical immunosuppressive effects at different concentrations of both chemicals in CD1® which gives approximation to decide the doses as positive control in immunotoxicity studies. Moreover, method

followed in this research identifies the minimum detectable counts of lymphocytes for comparative assessment of the results.

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