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Quality control of various laboratory mice by immunophenotyping approach by multicolor flow cytometry

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Abstract

Immunophenotyping by flow cytometry is one of the most rapid way of doing analysis & identification of heterogeneous populations of cells by using cell-specific fluorochrome-conjugated antibodies as probes. Immunophenotyping of laboratory animals is essential to monitor the immune status of the laboratory animals for better maintenance and management of animal facilities for immunological studies. The spleen is one of the major organs in immunity and plays a key role in the production and maintenance of red blood cells and the production of certain circulating white blood cells. Therefore, we harvested the spleen from various mice strain (Swiss Albino, BALB/C, C57BL, and C3HeJ) available at Central Animal Facility of Indian Institute of Science and did quantification of various types of immune cells by multicolor flow cytometry. $1X10^7$ splenocytes were taken in the eppendorf tubes and labeled with T cell, B cell, $CD4^+$ and $CD8^+$ cells specific antibodies and 30,000 events were acquired in the BD FACS Canto™ II (BD Biosciences) and the results were analyzed by FACS Diva software version 6.1.1. It was found that C57BL has higher percentage of B Cells (60±10%) followed by C3HeJ (49±2%). BALB/C has higher number of T cells (54±4%) followed by Swiss Albino strain (46±4%). BALB/C has higher percentage of $CD4^+$ Cells (39±3%) as compared to other strains. In most of the strains the B Cells population is more followed by T Cells, $CD4^+$ Cells & $CD8^+$ Cells. Moreover, splenocytes percent profile of Scid and Nude mice were taken as standard.

Key words: immunophenotyping, flow cytometry, mice

Introduction

Immunophenotyping is the analysis & identification of heterogeneous populations of cells by using fluorochrome-conjugated antibodies as probes to proteins expressed by the respective cells for the purpose of identifying the presence and proportions of various cell populations. Immunophenotyping using flow cytometry has become the method of choice in identifying and sorting cells within complex populations like analysis of T cells and B cells. In the last 20 years, there has been a steep increase in flow cytometry based immunophenotyping applications in the clinical laboratory and basic research with the availability of monoclonal antibodies rendering it less expensive, and user-friendly (Calvelli *et al.,* 1993; Bertram *et al.,* 2001; Cook *et al.,* 2003; Amatya *et al.,* 2004; Laane *et al.,* 2005; Rawstron *et al.,* 2006). With the discovery of cell specific surface antigenic markers and respective antibodies it has been easy to estimate the various cell types in the heterogeneous cell population (Lai *et al.,* 1998). Immunophenotyping can be used as a tool for immunological profiling and genetic monitoring of the various mouse strains as leukocyte alloantigens like CD45, CD90, IgM and major histocompatibility complex (MHC) molecules which are polymorphic across various mice strains. Immunophenotyping of laboratory animals is essential to monitor the immune status of the laboratory animals for better maintenance and management of animal facilities. Spleen is one of the major organs involved in immune system and plays a key role in the production and maintenance of red blood cells, the production of certain circulating white blood cells (Mebius and Kraal, 2005). Moreover, the spleen is also a site

for immune function, since it contains lymphatic tissue and produces lymphocytes. Lymphocytes are one of the five kinds of white blood cells or leukocytes, circulating in the blood. The most abundant lymphocytes are B lymphocytes and T lymphocytes and the sub set of T lymphocyte are $CD8^+$ T cells and $CD4^+$ T cells (Mebius and Kraal, 2005). Moreover, percent distribution of T Cells and B Cells in spleen is an indicative of Molony virus infection (Mertens and Krueger, 1976). Apart from the estimation of percent distribution of T cells and B Cells in the spleen, the positive B Cells or T cells can be sorted and used for further analysis by flow cytometry. Though there are other protocols for enrichment for various lymphocytes, like Miltenyi methods (Miltenyi Biotec, Auburn, CA). Therefore, we harvested the spleen from various strains of mice available at Central Animal Facility of Indian Institute of Science and did quantification of various types of immune cells by flow cytometry.

Animals

Male mice (20-25 g) of various strains (Swiss Albino, BALB/c, C57BL, and C3HeJ**)** were kept in polypropylene cages and maintained at 22 ± 2°C, 12 h day/night cycle and were provided with chow pellets and water *ad libitum*. All the animals were of 2 months age. Prior approval from the Institutional Animal Ethics Committee (IAEC) was obtained and the procedures were conducted as per CPCSEA guidelines.

Materials and methods

The NH_4Cl, $KHCO_3$, EDTA, Dulbecco′s Modified Eagle′s Medium (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS) were procured from Sigma-Aldrich, Inc (USA). The antibodies Anti-Mouse CD3e FITC, Armenian Hamster IgG Isotype Control FITC, Anti-Human/Mouse CD45R (B220) PE, Rat IgG2a K Isotype Control PE, Anti-Mouse CD8b PE-Cy5 and Anti-Mouse CD4 eFluor® 450 were procured from eBiosciences, Inc. (San Diego, USA)

Splenocytes isolation

The splenocytes were isolated with slight modification of protocol as described by Liu *et al.,* 2008 and Barclay *et al*., 1999. The animals were sacrificed under ether euthanasia and spleen was dissected out into a petri dish containing 5 ml of serum free DMEM media. It was crushed with a syringe plunger. The debris was removed with pipette-man and the suspension was filtered through a nylon mesh into 15ml tube. The tubes were centrifuged at 1500 rpm for 5 min at 4°C. The pellet was suspended in 5ml ACK lysis buffer for 1 min followed by addition of 10ml serum free DMEM media. It was further centrifuged 1200 rpm for 5 min at 4°C. Lysis step was repeated if the pellet was red. The pellet should be white. The white pellet was re-suspended in DPBS.

Immunolabeling and Flow Cytometry

$1X10^7$ cells were taken in the eppendorf tubes and labeled with antibodies (Anti-Mouse CD3e FITC, Armenian Hamster IgG Isotype Control FITC, Anti-Human/Mouse CD45R (B220) PE, Rat IgG2a K Isotype Control PE, Anti-Mouse CD8b PE-Cy5 and Anti-Mouse CD4 eFluor® 450) as per the manufacturers' instructions. It was acquired in the BD FACS Canto™ II (BD Biosciences) and the results were analyzed by FACS Diva software version 6.1.1.

Statistical analysis

Values have been reported as the Mean±SEM in all the groups. Comparisons between the different groups were performed by One-way ANOVA test and differences were considered significant at $p<0.05$.

Results

The percent of various cell types was estimated as shown in the dot plot obtained after flow cytometry analysis of splenocytes after labeling with Isotype control antibodies, different cell specific antibodies and all antibodies together (Fig-1). There was not much qualitative difference in the various splenocytes population of different mice strains as evident by dot plot analysis of flow cytometry data in the presence of all antibodies (Fig-2). Moreover, statistical analysis of the quantitative data showed that C57BL has higher percentage of B Cells (60±10%) followed by C3HeJ (49±2%) (Table-1). BALB/C has higher number of T cells (57±4%) followed by Swiss Albino strain (46±4%). BALB/C has higher percentage of CD4 Cells (42±3%) as compared to other strains (Table-1). In most of the strains the B Cells population is more followed by T Cells, $CD4^+$ Cells & $CD8^+$ Cells (Fig-2) (Table-1).

Discussion

The percentage of T cells & B Cells play crucial role in various pathogenesis of diseases as suggested by various experiments conducted in mice. $CD4^+$ and $CD8^+$cells have been implicated in the pathogenesis of Multiple sclerosis, which is an inflammatory disease of the central nervous system. Multiple sclerosis is characterized by multi-focal demyelination, axonal loss, and immune cell infiltration (Mars *et al.,* 2011). In disease conditions like Non Obese Diabetic mice, CD8+ T cells is significantly increased in the intrahepatic lymphocyte fraction of NOD and there is more formation of IFN-γ and TNF-α, compared with controls (Yang *et al*., 2011).

Moreover, increase or decrease in the level of various T cells and B cells increases the susceptibility to various diseases and the percentage of the cell populations alters during various disease conditions as suggested by various studies (Mars *et al.,* 2011; Yang *et al*., 2011; Zhou *et al.,* 2011).

Moreover, we assessed the splenocytes percent profile of nude mice and found that 88% of the splenocytes were observed to be B cells in the nude mice whereas T cells were totally absent or negligible (0.9%) in the nude mice (Fig-3). We also assessed the percent population of various splenocytes in Scid mice. However, there was neither T cells nor B cells present in Scid splenocytes (Fig-4).

Moreover, various cells like $CD8^+CD122^+$ regulatory T cells (Tregs) play crucial role in maintaining immune homeostasis. Endharti *et al.,* (2011) have shown that $CD8^+CD122^+$ Tregs effectively prevent and cure colitis in a mouse model. They show the potential role of $CD8^+$ Tregs, and possibly together with $CD4^+$ Tregs, in the medical care of inflammatory bowel disease patients (Endharti *et al.,* (2011). $CD4^+$ T cells have been found to play key role along with endothelial cell, macrophages and $CD4^+$ T cells in preventing cerebrovascular amyloid deposition (Weiss *et al.,* 2011). Moreover, various infections like Intraperitoneal *Echinococcus multilocularis* infection in mice modulates peritoneal $CD4^+$ and $CD8^+$ regulatory T cell development (Mejri *et al*., 2011). Many studies are there, where they have taken percentage increase or decrease of T cells from spleen as an immune parameter in considering the effect of various treatments. Dreau *et al.*, (2000) have observed the there is increase in CD3 and TCRalpha expressing cells following 2-deoxy-D-glucose administration in BDF_1 mice (Dreau *et al.*, 2000).

Alteration in the normal percentage of splenocytes is observed in chronic lymphocytic leukemia in mice (Santanam *et al.,* 2010). Moreover, T cells percentage are also modulated following infection with Lymphocytic Choriomeningitis Virus and Influenza Virus (Mueller *et al*., 2010). Moreover, dietary conditions like vitamin A-deficiency alter the lymphocyte populations in the spleen. It has been observed that $CD4^+$ T cells decreased whereas B cells and $CD8^+$ T cells numbers remained unaffected in vitamin A-deficient mice (Duriancik and Hoag, 2010). It has been found that *P. aeruginosa* infections in cystic fibrosis patients increases the proliferation of T cells and B cells leading to immunoinflammatory response in the patients causing higher morbidity and mortality (Barclay *et al*., 1999).

Conclusion

The results show that there is variation in the percentage of immune cell types across various mice strains, though there are not significant differences among various strains. Therefore, the immune reactions may differ in various mice strains in various experimental studies. Therefore, proper mice strains are to be selected for various studies. Moreover, there is no information available on the percentage of various immune cell types across various mice strains. Therefore, this study will help in bridging the information gap and provide valuable information to the researcher involved in immune cell studies in various mice strains.

References

Amatya R, Vajpayee M, Kaushik S, Kanswal S, Pandey RM, Seth P (2004). Lymphocyte immunophenotype reference ranges in healthy Indian adults: Implications for management of HIV/AIDS in India. *Clin. Immunol.* 112: 290-295.

Barclay NG, Spurrell JC, Bruno TF, Storey DG, Woods DE, Mody CH (1999). *Pseudomonas aeruginosa* exoenzyme S stimulates murine lymphocyte proliferation in vitro. *Infect. Immun.* 67: 4613-4619.

Bertram HC, Check IJ, Milano MA (2001). Immunophenotyping large B-cell lymphomas. Flow cytometric pitfalls and pathologic correlation. *Am. J. Clin. Pathol.* 116: 191-203.

Calvelli T, Denny TN, Paxton H, Gelman R, Kagan J (1993). Guideline for flow cytometric immunophenotyping: A report from the National Institute of Allergy and Infectious Diseases, Division of AIDS. *Cytometry*. 14: 702-715.

Cook JR, Craig FE, Swerdlow SH (2003). bcl-2 Expression by multicolor flow cytometric analysis assists in the diagnosis of follicular lymphoma in lymph node and bone marrow. *Am. J. Clin. Pathol.* 119: 145–151.

Dréau D, Foster M, Morton DS, Fowler N, Kinney K, Sonnenfeld G (2000). Immune alterations in three mouse strains following 2-deoxy-D-glucose administration. *Physiol. Behav.* 70:513-520.

Duriancik DM, Hoag KA (2010). Vitamin A deficiency alters splenic dendritic cell subsets and increases CD8(+)Gr-1(+) memory T lymphocytes in C57BL/6J mice. *Cell Immunol.* **265:**156-163.

Endharti AT, Okuno Y, Shi Z, Misawa N, Toyokuni S, Ito M, Isobe K, Suzuki H (2011). CD8+CD122+ regulatory T cells (Tregs) and CD4+ Tregs cooperatively prevent and cure CD4+ cell-induced colitis. *J. Immunol.* 186:41-52.

Laane E, Tani E, Bjorklund E *et al.* (2005). Flow cytometric immunophenotyping including Bcl-2 detection on fine needle aspirates in the diagnosis of reactive lymphadenopathy and non-Hodgkin's lymphoma. *Cytometry B Clin. Cytom.* 64B: 34-42.

Lai L, Alaverdi N, Maltais L, Morse HC (1998). 3rd. Mouse cell surface antigens: nomenclature and immunophenotyping. *J. Immunol.* 160 : 3861-3868.

Liu C, Xi T, Lin Q, Xing Y, Ye L, Luo X, Wang F. Immunomodulatory activity of polysaccharides isolated from *Strongylocentrotus nudus* eggs. *Int. Immunopharmacol.* 8: 1835-1841.

Mars LT, Saikali P, Liblau RS, Arbour N (2011). Contribution of CD8 T lymphocytes to the immuno-pathogenesis of multiple sclerosis and its animal models. *Biohem. Biophys. Acta.* 1812:151-161.

Mebius RE, Kraal G (2005). Structure and function of the spleen. *Nat. Rev. Immunol.* 5 : 606-616.

Mejri N, Müller N, Hemphill A, Gottstein B (2011). Intraperitoneal *Echinococcus multilocularis* infection in mice modulates peritoneal CD4+ and CD8+ regulatory T cell development. *Parasitol. Int.* 60:45-53.

Mertens H, Krueger GRF (1976). Percent Distribution of T- and B-Lymphoid Cells in Spleen and Lymph Nodes of Moloney Virus Infected Mice. *Z. Krebsforsch.* 85: 169-175.

Mueller SN, Langley WA, Li G, García-Sastre A, Webby RJ, Ahmed R (2010). Qualitatively different memory CD8+ T cells are generated after lymphocytic choriomeningitis virus and influenza virus infections. *J. Immunol.* 185:2182-2190.

Rawstron AC, de Tute R, Jack AS, Hillmen P (2006). Flow cytometric protein expression profiling as a systematic approach for developing disease-specific assays: identification of a chronic lymphocytic leukaemia-specific assay for use in rituximab-containing regimens. *Leukemia.* 20: 2102–2110.

Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, Volinia S, Alder H, Rassenti L, Kipps T, Croce CM, Pekarsky Y (2010). Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. *Proc. Natl. Acad. Sci.* 107:12210-12215.

Weiss R, Lifshitz V, Frenkel D (2011). TGF-β1 affects endothelial cell interaction with macrophages and T cells leading to the development of cerebrovascular amyloidosis. *Brain Behav. Immun.* 25:1017-1024.

Yang GX, Wu Y, Tsukamoto H, Leung PS, Lian ZX, Rainbow DB, Hunter KM, Ridgway WM (2011). CD8 T cells mediate direct biliary ductule damage in non-obese diabetic autoimmune biliary disease. *J. Immunol.* 186: 1259-1267.

Zhou Z, Niu H, Zheng YY, Morel L (2011). Auto reactive marginal zone B cells enter the follicles and interact with CD4+ T cells in lupus-prone mice. *BMC Immunol.* 12:7.

Table-1: Percentage of B Cells, T Cells, CD4 Cells & CD8 Cells in various strains of mice

Mice Strain	B-Cell (%)	T-cell (%)	CD4 Cells (%)	CD8 Cells (%)
Swiss Albino n=3	***45±5***	***46±4***	***35±3***	***12±0.4***
BALB/c n=4	***37±3***	***54±4***	***39±3***	***14±1***
C57BL n=3	***60±10***	***34±10***	***25±8***	***9±1***
C3HeJ n=3	***49±2***	***39±2***	***30±3***	***11±0***
Nude n=1	***88±0***	***0.9±0***	***0±0***	***0.9±0***
Scid n=1	***0±0***	***0±0***	***0±0***	***0±0***

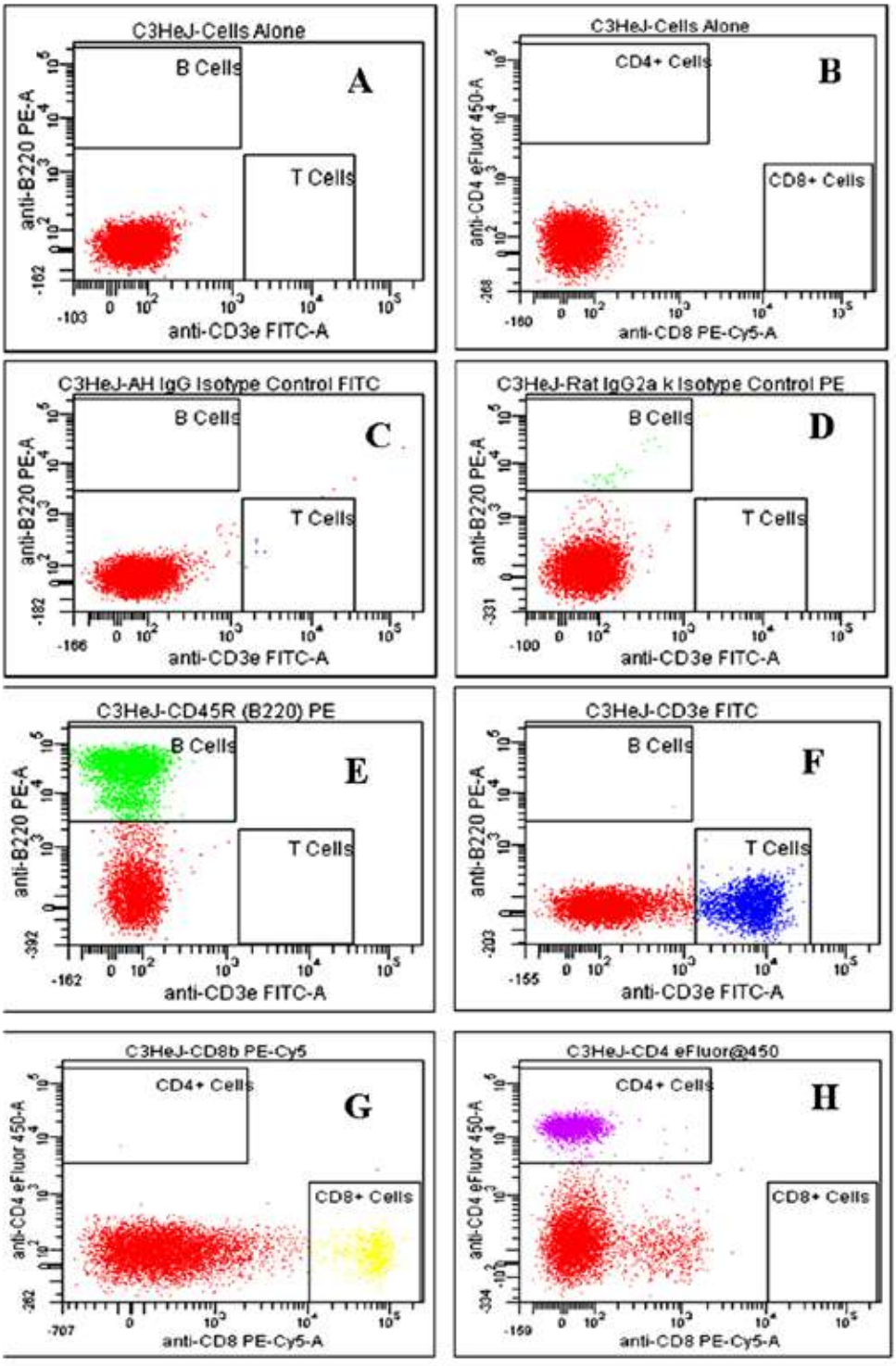

Fig-1: Dot plot of Flow cytometry analysis of the splenocytes

A : Control cell population for B Cells & T Cells.
B : Control cell population for $CD4^+$ & $CD8^+$ Cells.
C & D: Shows labeling with Armenian hamster IgG Isotype Control FITC & Rat IgG2a k Isotype control PE respectively.
E : T Cell populations after labeling with T Cells specific antibodies.
F : B Cell populations after labeling with B Cells specific antibodies.
G & H : $CD4^+$ Cells & $CD8^+$ Cells after labeling with $CD4^+$ Cells & $CD8^+$ specific antibodies respectively.

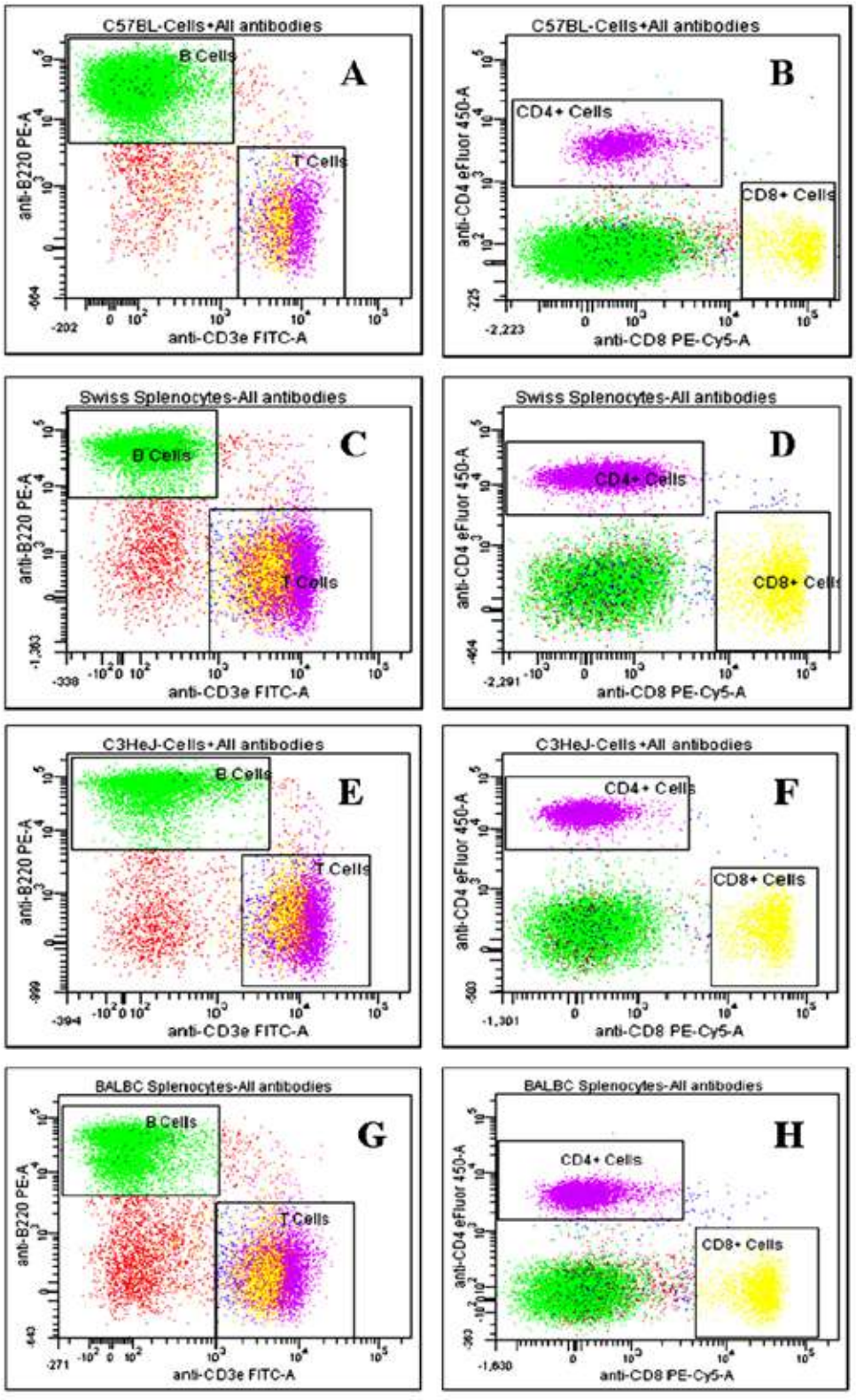

Fig-2: Dot plot of various splenocytes percent profile in different mice strains.
A & B for C57BL; C & D for Swiss; E & F for C3HeJ; G & H for BALB/C.

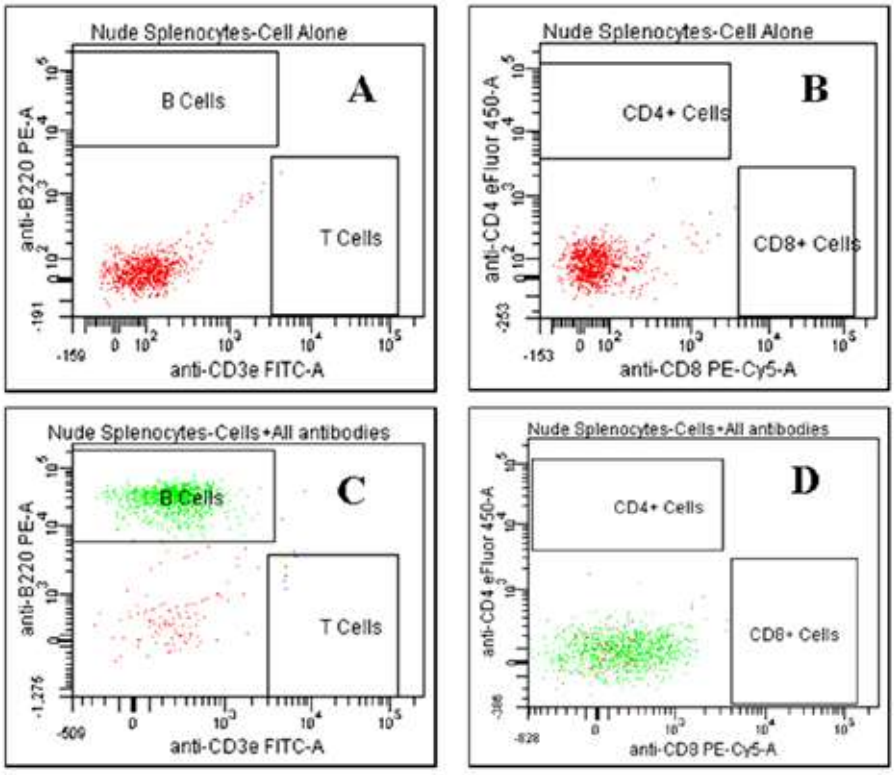

Fig-3: Splenocytes per cent profile in Nude mice. .

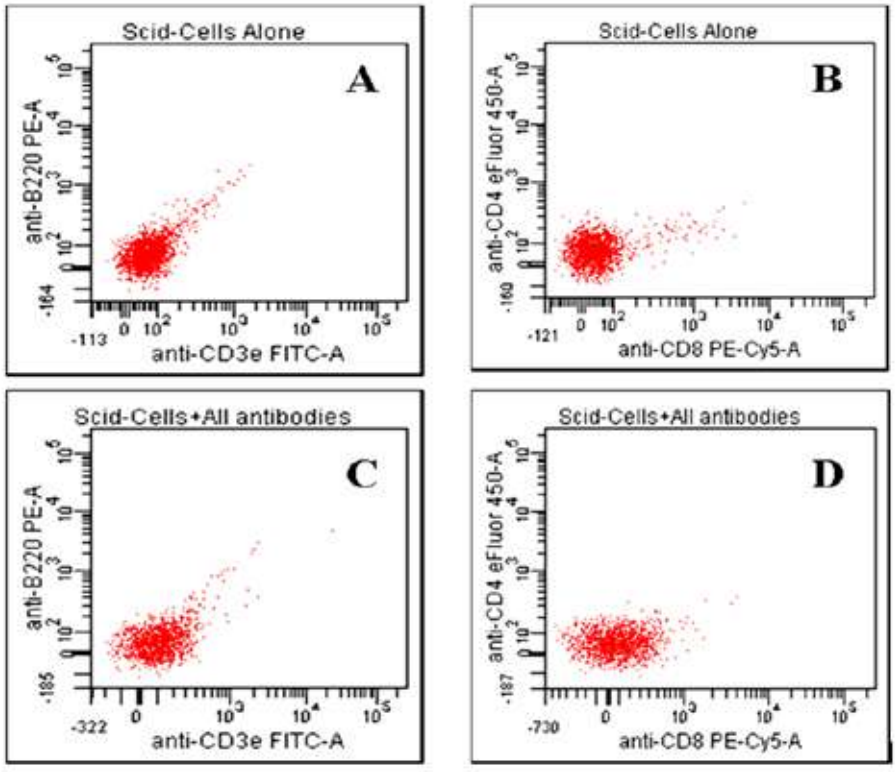

Fig-4: Splenocytes per cent profile in Scid mice.