RESEARCH ARTICLE

Editorial Process: Submission:06/26/2017 Acceptance:12/12/2017

Differential Response of B Cells to an Immunogen, a Mitogen and a Chemical Carcinogen in a Mouse Model System

Nimisha Saxena, Amar Preet Kaur, Nimai Chand Chandra*

Abstract

Background: B cells are specific antibody generating cells which respond to foreign intruders in the circulation. The purpose of this study was to compare the relative immunogenic potentials of three well established agent types viz. an immunogen, a mitogen and a carcinogen, by following B cell responses to their presence in a mouse model system. **Methods:** Mice were treated with tetanus toxoid (immunogen), poke weed mitogen (typical mitogen), and benzo-α-pyrene (carcinogen) and generated B cell populations were determined in isolated splenic lymphocytes (splenocytes) by flow cytometry using specific anti-B cell marker antibodies. Flow cytometric estimation of LDL receptor (LDLR) expression, along with associated B cell markers, was also conducted. Kit based estimation of serum IgG, western blotting for LDLR estimation on total splenocytes and spectrometry for cholesterol and serum protein estimation were further undertaken. Student's T-tests and one way ANOVA followed by the Bonferroni method were employed for statistical analysis. **Results:** The mitogen was found to better stimulate B cell marker expression than the immunogen, although the latter was more effective at inducing antibody production. The chemical carcinogen benzo-α-pyrene at low concentration acted potentially like a mitogen but almost zero immunity was apparent at a carcinogenic dose, with a low profile for LDLR expression and intracellular cholesterol. **Conclusion:** The findings in our study demonstrate an impact of concentration of BaP on generation of humoral immunity. Probably by immunosuppression through restriction of B-cell populations and associated antibodies, benzo-α-pyrene may exerts carcinogenicity. The level of cholesterol was found to be a pivotal target.

Keywords: B cells- IgG- LDL receptor- cholesterol- tetanus toxoid- pokeweed mitogen- benzo- alpha- pyrene

Asian Pac J Cancer Prev, **19 (1)**, 81-90

Introduction

In the history of medicine, an event of molecular recognition that could discriminate between self and non-self with an ingenuous power to eliminate only the non self, finally reckoned as a shield against pathogens and got entitled as host immune response.

The immune response is involved to protect multicellular organisms through elimination or neutralization of the invaders that are normally called antigens (Lindenmann, 1984). The typical immunogenic antigens possess a mitogen like response on B cell proliferation along with the secretion of its active ingredient(s) popularly called the antibody (Skidmore et al., 1975) but in case of carcinogenic invaders, the scenario is different. Though potentially a carcinogen behaves like the mitogen, the mechanism by which a carcinogen skips the immune buffer, inactivates antibody generating machinery and ultimately leads to carcinogenesis, is still not very transparent (Headley et al., 1977; O'Byrne et al., 2000).

Since immune response is a property of plasma lymphocytes (Balakrishnan and Adams, 1995) and basically a cognitive attack on foreign intruders; the population of activated immune cells would be the only paramount armor in such occasions. Antibodies are major partners in immune defense mechanism and their plethora is only obvious by expediting the activated B-cell reproduction (Janeway et al., 2001). Ironically, in immune defense mechanism, the immunogen induced B-cell proliferation apparently matches the chemistry of mitogen promoted cell proliferation (Bryan and Norris, 2010). Though, it is obviously not transparent whether the immunogens are fully compatible with a typical mitogen (e.g. pokeweed)(Pahwa et al., 1981; Jones , 1983) or mitogenic activity of a carcinogen (e.g. benzo-alpha-pyrene) (Matiasovic et al., 2008), it is at least obvious that immunogens may give a lead to antibody production and their secretion into circulation through proliferation of B-cells. Though there is a strategic difference in the principle of gene expression in normal cell proliferation by the mitogens and secretion of antibody after B-cell proliferation by immunogens; the template of execution by both partners apparently follows the base line principle, primarily the cell multiplication.

Mitogens stimulate cell growth which is a cumulative success of many intracellular and extracellular enactments

*Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India. * For Correspondence: nc1_chandra@hotmail.com*

(Saxon, 2004). Although classically cholesterol is an essential complement in the scaffold of biological membrane (Albi and Magni, 2004); recent advents have also shown cholesterol as an invaluable participant in the process of cell proliferation (Fernández et al., 2005; Sun et al., 2014; Viola-Magni and Gahan, 2012; Cascianelli et al., 2008). Reports from our (Verma and Chandra, 2016; Sankanagoudar et al., 2017; Pandey et al.,online version ; Singh G et al., 2017) and other (Albi E and Magni M , 2004; Albi E ,2011) laboratories have shown the passage of cholesterol into cell nucleus generating interest for forth coming survey of the status of cholesterol in cell nucleus and cell proliferation. Following 1980's the role of cholesterol on the regulation of cell cycle component was observed in both cancer and growing cells (Rao ,1986; Singh et al., 2013; Martínez-botas et al.,1999). Reports have shown the presence of cholesterol in chromatin structure and nuclear matrix (Cascianelli et al., 2008; Martínez-botas et al.,1999; Albi et al., 2003; Lajtha et al., 2007; Kolomiytseva et al., 2016).

Experimental evidence indicates that exponential cell proliferation (Bensinger et al., 2008; Lo Sasso et al., 2010) and tumor growth (Clendening et al., 2010; Dang, 2012) are closely associated with enhanced cholesterol requirement. Some types of cancers, such as hepatocellular carcinoma (HCC), are dependent on cholesterol for growth (Borena et al., 2012). In hypercholesterolemia, cholesterol reaches the nuclear lipid microdomains where it activates gene expression (Codini and Cataldi, 2016). (Srere et al.,1948) reported that rapidly growing tissues such as the brains of newborn rats actively synthesized cholesterol from two-carbon fragments; whereas slow growing adult brain carried out only slow rate of de novo cholesterogenesis. Zhenyi and William (2016) and Villa et al., (2016) reported that depriving deadly brain cancer cells of cholesterol, which they import from neighboring healthy cells, specifically kills tumor cells and caused tumor regression. This is to note that intracellular cholesterol homeostasis is maintained by joint sharing of cellular biosynthesis, LDL–receptor (LDLR) mediated endocytosis and exocytosis of VLDL (Lange and Steck, 1996; Soccio and Breslow, 2004; Brown and Goldstein, 1986; Fabiola et al., 2015).

Studies on lipid metabolic reprogramming (lipidomics) in cancer cells is now an immerging target in cancer biology (S Beloribi-Djefaflia et al., 2016) to improve chemotherapy, radiotherapy and immunotherapy in cancer treatment protocol (Zalba and Ten Hagen, 2017). Clinical and experimental reports (Clendening JW et al., 2010; Dang, 2012) in recent past have shown that the finely tuned mechanisms of intracellular cholesterol homeostasis become altered during cell division and membrane synthesis both in physiological hyperproliferative conditions and in carcinogenesis. The specific regions within the lipid rafts, present as micro-domains in cell membrane are associated with several proteins that regulate pro-oncogenic and apoptotic pathways and are involved in the initial stages of cancer development, tumor growth and the potential progression to a migratory and metastatic phenotype (Yang et al., 2014).

Siperstein et al., (1966) showed a negative feedback control of cholesterol biosynthesis in transplantable hepatomas. Bricker and Levey (1972) demonstrated unsurpassable cholesterol and fatty acid synthesis by cyclic nucleotides in hepatomas indicating a deletion of their regulatory mechanism of lipogenesis. In normal cells LDLR expression is regulated by a sterol regulatory element (SRE-1) located at -65/-56 of ldlr gene (Brown and Goldstein, 1975; Ho et al.,1976; Havekes et al., 1987; LaVoie et al., 1999; Smith et al., 1990; Brown and Goldstein,1997), but cancer cells are obviated from such regulation (Tatidis et al., 1997). Diminution of feedback regulation of LDLR has been reported in the Daudi Burkitts lymphoma cells (Chen and Hughes-Fulford , 2001) and in an acute myelogenous leukemic cells from patients (Tatidis et al., 1997). These studies also showed elevated activity of LDLR in patient's leukemic cells compared to normal white blood cells. It is also known that mitogen increases the LDLR mRNA concentration (Sanctis, 1998; Cuthbert and Lipsky, 1990).

LDL receptor expression by mitogenic surge (in presence of insulin) has been found to be under control of sterol regulated feedback mechanisms in cells (Gopalakrishnan and Chandra , 2006). Chen et al., (1975) first showed that phytohemagglutinin-stimulated mouse lymphocytes underwent a six fold increase in acetate incorporation into cholesterol 24 h before the cells entered the S-phase of the cell cycle. Unlike biological mitogens the SREBP mediated feedback regulation remains declined in presence of carcinogens.

A global incentive on cancer research in prevailing time intends to understand the mechanism that allows a carcinogen to skip the immune barrier of host and helps its advancement for carcinogenesis. The present study is designed to explore the differential role of three components viz. immunogen , mitogen and carcinogen on B lymphocyte population and its secreted antibodies from the perspectives of immunological response and the stature of cellular cholesterol concentration. Basically we have pointed our aims to know how the mitogenic bout of carcinogens differ from that of immunogen or classical mitogen in the processing of immune cells particularly B lymphocytes targeted in present study.

Materials and Methods

Tetanus Toxoid was a gift from National Institute of Immunology, New Delhi, India. Pokeweed mitogen (Phytolacca Americana) and Benzo-alpha-pyrene were purchased from Sigma, St. Louis, USA. Freunds Complete and Incomplete Adjuvant were obtained from Difco laboratories, Detroit, Michigan 48232-7058 USA. RBC Lysis Buffer was procured from Bio basic inc. RPMI 1640 was bought from Sera laboratories international ltd. Fetal Calf Serum was delivered by biological industries, Kibbutz beithaemek 25,115 Israel. Fluorescent tagged antibodies for flowcytometry e.g. Anti-LDLR (BS-0705R) (PE-Cy7 tagged) was purchased from Bioss, Anti-CD79a (cat no-133103) (PE tagged) from Biolegend and Anti-CD19 (cat no-1101-93) (FITC tagged) was obtained from eBioscience.Coomassie brilliant blue R-250 was purchased from Bio-Rad laboratories, Hercules, USA.

Acrylamide, bis-acrylamide and Colorburst / Fermentas protein molecular weight standards were purchased from Sigma, St. Louis, USA. Nitrocellulose membrane was a product of Genotech Incorporation, CA, USA. Secondary antibodies (anti goat and anti mouse) HRP conjugates were from Biotechnology Inc., Santa Cruz,CA,USA. ECL reagent (Enhanced Chemi Luminescence) was obtained from Santacruz Biotechnologies, CA, USA. Kodak XK-5 X-Ray films were got from Kodak corporation, USA. Mouse IgG Elisa kit [E.Bioscience.USA.(cat no 88.50400-22)] was used for substrate and secondary antibody for IgG estimation on an antigen precoated Elisa plate.

Female Swiss Albino mice were issued from Central Animal Facility of AIIMS, New Delhi, India, after ethical clearance. Rest of the materials were from local purchase.

Methods

Ethics approval and consent to participate

Animal experiments were performed in this study after receiving written consents from the Institute Body for Animal Ethical Clearance (Ref. No: 589/IAEC/11. Date: 17.06.2011).

Animals

Female Swiss Albino mice, 5 to 6 weeks old, were housed with color-mark (four animals/cage) to specify their identity for specific groups. They were quarantined for at least one week before used for experimentation. During this interval, they attained 17 to 20 g body weight. The animals were housed with sawdust bedding having tap-water and Purina (rodent chow) supply to dine.

Treatment of mice

Mice were marked into three groups having five mice per group. Each group maintained its own control. Each batch of experiment was repeated six times. Mice were treated with Tetanus Toxoid (TT) [Group-A, immunogen gr.], Poke weed mitogen (PWM) [Group-B, mitogen gr.] and Benzo alpha pyrene (BaP) [Group-C, mito-carcino gr.]. Control groups were treated with vehicles (PBS for Gr.-A and –B; Corn oil for Gr.-C respectively). TT was reconstituted in 1X PBS with Freund's Complete and Incomplete Adjuvant; PWM in 1X PBS and BaP in corn oil respectively. In each case the material was given intra-peritoneal (i.p.) in multiple doses.

Treated dose

Injections were given on the zero-day (primary dose) per kg b.w. of mice for TT: 5, 10, 20, 25, 40µg, PWM: 5, 10, 25, 50, 100µg, and BaP: 25, 50, 100, 150, 200, 250mg respectively. On 10th day, half of the above mentioned doses were injected by i.p. in each group as booster. Except others, booster of TT was reconstituted in 1X PBS with Freund's Incomplete Adjuvant. On 21st day mice were sacrificed by cervical dislocation.

Lymphocyte isolation

Spleens were removed aseptically from all mice of a particular group (control, TT, PWM, BaP) and pooled together. Cell suspensions were prepared from spleens by gentle teasing with forceps in Petri plates containing 10ml of RPMI-1640 and centrifuged at 2000rpm for 10min at room temperature. Pellet was dissolved in 5 ml of RBC lysis buffer (1X), kept for 15 min and washed twice with RPMI-1640. Cells were then re-suspended in 2 ml of RPMI-1640 having 10% fetal calf serum fortified with1% antibiotic-antimycotic and counted in Neubar's chamber. Cell viability was determined by the trypan blue exclusion method .Count of maximum number of viable cells for a given dose was the determinant for selecting the optimal in vivo dose of tetanus toxoid (maximum response with 25µg/kg), poke weed mitogen (maximum response with 10µg/kg) and BaP [25mg/kg (max. response) and 100mg/kg (carcinogenic dose)] in this study.

Flowcytometry

10^6 cells after counting in Neubar's chamber were taken in an eppendorf tube. Cells were kept in ice for 30 min and centrifuged at 2,000 rpm for 10 min at 4°C. Supernatant was discarded and pellet was rinsed twice with 200µl of 5% PBS-BSA and dissolved in 100µl PBS-BSA. Antibodies (anti CD79a, anti CD19, antiLDLR Phycoerythrin / FITC ,PE-Cy7 tagged respectively) were added at appropriate concentrations standardized beforehand. After 1hr. incubation, cells were washed twice with 5% PBS-BSA, dissolved in PBS-BSA and fixed in Paraformaldehyde. Instrument details: Model no: FACSCANTO Serial no: V07300557.

Cell lysis

Lymphocytes were isolated from mice spleen as mentioned above and washed in ice chilled 1X PBS twice at 2500 rpm for 5 min at 4°C. Lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-HCL (pH 7.6), 5 mM EDTA and 10 mM Iodoacetamide) containing protease inhibitor (PMSF: 100 nm, Leupeptine: 10µL, Aprotinin: 10µL) was added 5 times the volume of cell pellet and incubated for 30 min in ice followed by centrifuged at 12,000 rpm for 15min at 4◦C. The supernatant was stored at 4°C for protein estimation and western blot analysis.

Protein estimation

Protein was estimated in the cell lysate following the procedure of Bradford MM,1976.

SDS-PAGE

SDS – PAGE was carried out on 1.5 mm thick gels. The resolving gel was composed of 10% acrylamide and was overlaid by a 4% acrylamide stacking gel. The lysate containing 50 -60µg protein were mixed with an appropriate volume of 5x protein loading buffer containing 1% SDS, 5% β-mercaptoethanol, 50mM Tris-HCl (pH= 6.8), 10% Glycerol and 0.02% Bromo Phenol Blue and then boiled for 5 minutes. The samples were then brought down to room temperature before loading. Electrophoresis was performed at 10 mA – 12 mA till the dye front passed down into the electrophoresis buffer.

Western Blotting

The proteins were transferred from SDS-PAGE onto

nitrocellulose membrane. Transfer was performed at 35 V for 16 hours at 4°C in a transfer buffer containing 48mM Tris Base, 39 mM Glycine, and 20% Methanol, pre-chilled at 4°C. The membrane was incubated with blocking buffer [1.5% BSA in 0.01M PBS containing 0.05% Tween-20] for 2 to 4h at room temperature on a gel rocker and then washed thrice in 0.01 M PBS–0.1% Tween-20, for 10 min each. Anti LDLR goat polyclonal antibody, anti SREBP-2 goat polyclonal antibody, and β-actin mouse monoclonal antibody diluted in 0.01 M PBS–0.1% Tween-20 were added at 1:2000 or 1:500, dilutions respectively as per the appropriate blots and the membrane was incubated at room temperature for 2 hours. The membrane was then washed thrice in 0.01 MPBS–0.1% Tween-20 for 10 min each. Anti-goat rabbit HRP conjugate anti-antibody was added at 1: 8000 for LDLR, 1:3000 for SREBP-2 and anti-mouse-HRP (1:2000) for β Actin anti-antibody and incubated for 2 hours at room temperature. This was followed by three washes in PBS–Tween-20 buffer. The blots were then developed by Enhanced Chemi Luminescence in the dark and the bands were captured on a Kodak XK-5 X-Ray film. Alpha tech imager was used for the analysis of intensity of the respective bands.

Serum IgG Estimation

Kit based estimation of IgG produced by antigen exposure was performed in serum separated from the blood collected on 21st day of 1st antigen application. In brief, antigen (TT, PWM and BaP) was coated at concentration of 100ng/100µl on 96 well ELISA plate and incubated overnight. Wells were washed four times with wash buffer, blocked with milk powder after incubation of 1h and washed again as above. Serum was then added at serial dilution and incubated for duration of 2h. Followed by three washes, enzyme-antibody conjugate was added into the wells at appropriate concentration and incubated for 30 min in dark at room temperature. After appropriate washing, TMB-substrate(3,3´ 5,5´Tetramethylbenzidine) was added and incubated in dark for 10min. The reaction was stopped by adding stop solution. Reading was taken at 450 nm.

Cholesterol estimation

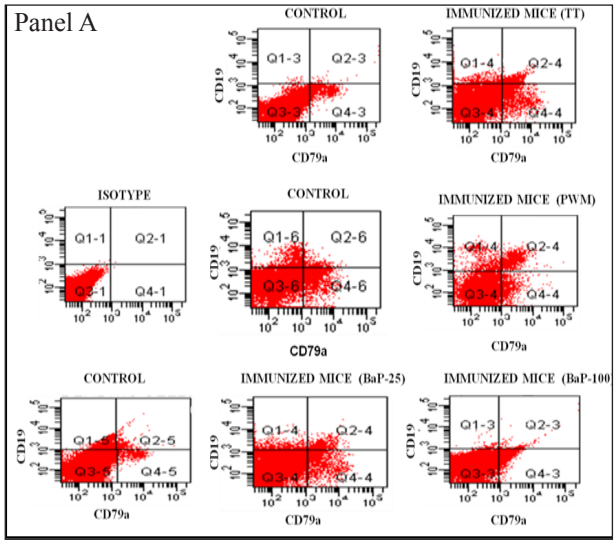
Cholesterol was estimated by following the protocol of Zlatkis et al.,(1953). ($FeCl_3$-CH_3 COOH).

Statistical analysis

p-values were calculated by Student's T-test and one way ANOVA (post Hoc tests) followed by Bonferroni method.

Results

Percentages of B-cell populations, increased by inducers e.g. TT, PWM and BaP has been compared in Figure 1. Percentages of two B-cell specific CD markers viz CD19 and CD79a in combination have been verified by flow cytometry (Figure 1A) in presence of three inducers. While the effects of three inducers have been compared for combination CD markers against their respective controls, it was transparent that the relative increase of B-cells by



Panel B

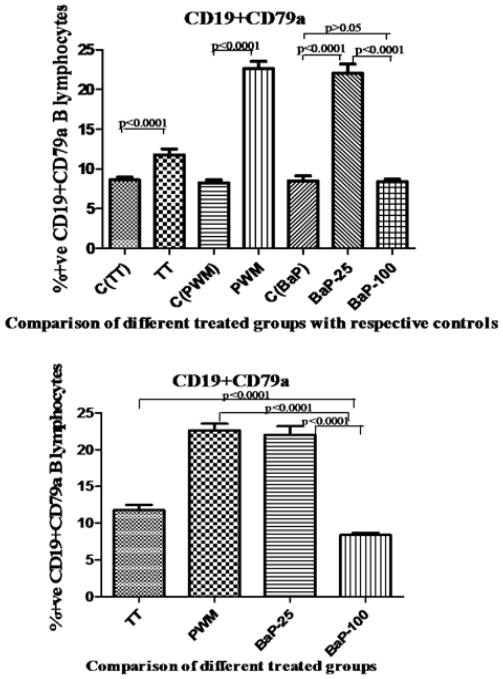
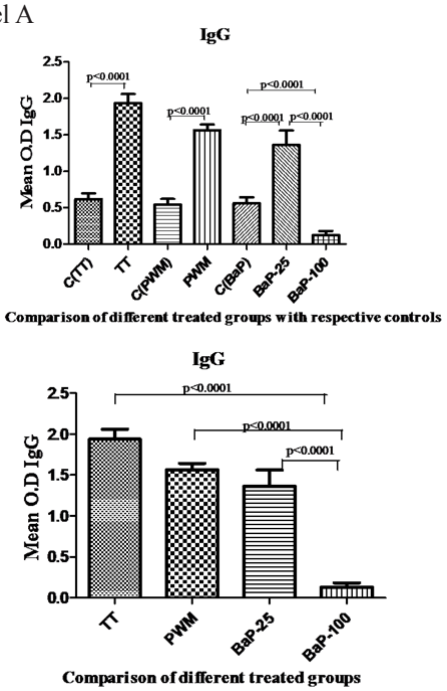


Figure1. Relative expressions of B cells by TT, PWM and BaP. Panel-A, Representation of Flow Cytometry for CD19 and CD79a (B cell markers) labeled cells; Panel-B, Top, Bar graphs showing relative potentiality of immunogen, mitogen and carcinogen on the expression of B-cells evaluated with cell specific combination markers CD19 + CD79a; Bottom, Shows comparison on different treated groups; Respective p-values between the pairs are shown on the top of the bars.

PWM and BaP25 were comparatively higher than the increase of B-cells shown by TT (Figure 1B top-panel). This indicated relatively more mitogenic propensity of PWM and BaP25 as compared to TT. Apparent cell death or cytotoxicity in B-cell population has been found to initiate at the onset limit of carcinogenic dose of BaP viz BaP100, and got extravagant at further higher doses (watched up to 250mg per kg b.w.). So we compromised our study keeping maximum BaP dose limited at BaP100. A gross comparison of the events has been presented with combination CD marker CD19 + CD79a (Figure

Panel A



Panel B

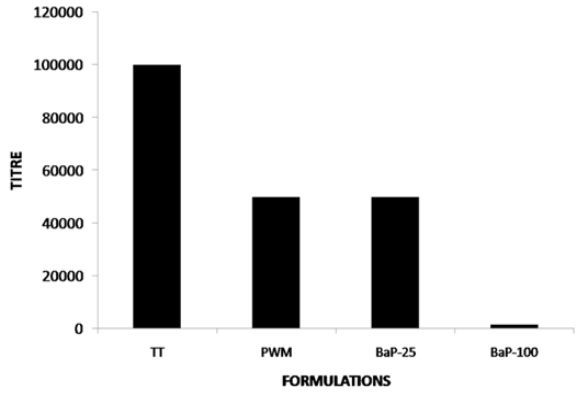
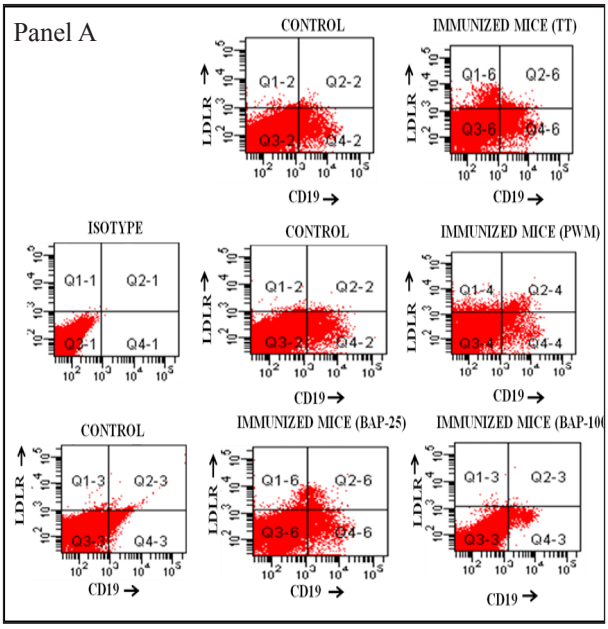


Figure 2. Serum IgG levels by TT, PWM and BaP. Panel-A, Top, Bar graphs showing relative response of immunogen, mitogen and carcinogen on serum IgG levels; Bottom, Graphs showing comparison on different treated groups; Panel-B, Comparison of antibody titre by respective concentrations of TT, PWM, Bap25, Bap-100 showing their relative immunogenic efficiency; Respective p-values between the pairs are shown on the top of the bars.

1B bottom-panel). This is to highlight that PWM and BaP25 were found as better cell proliferating agent (more mitogenic potential) lacking characteristic properties of a native immunogen like TT.

Although TT was found to be characteristically less mitogenic as compared to PWM and BaP25, it displayed its native immunogenic accountability (Figure 2A, bottom-panel) by showing relatively higher production of IgG as compared to PWM and BaP25 on similar exposure. This shows that PWM and BaP25 being a foreign intruder in mice system possess poor immunogenic potency as compared to a typical immunogen e.g. TT. Immunogenic response on antibody production is predominantly an expression of gene transcription rather than a production

Panel A



Panel B

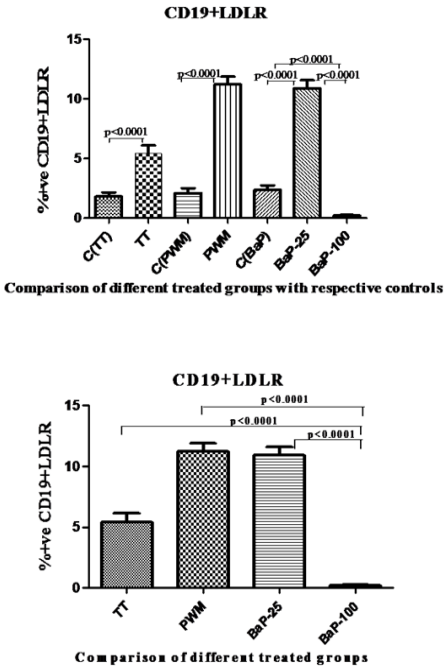
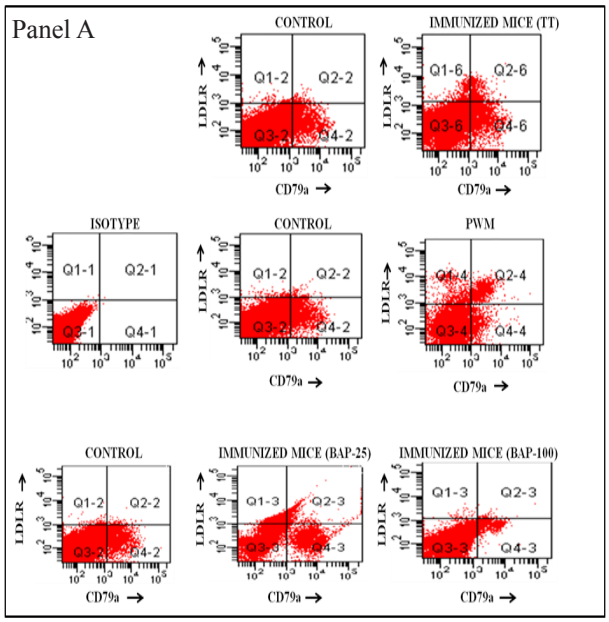


Figure 3. Relative Expressions of LDLR on CD19 labeled B-Cells by TT, PWM and BaP. Panel-A, Representation of Flow Cytometry for LDLR expression by CD19 B-cells; Panel-B, Top, Bar graphs showing relative potentiality of immunogen, mitogen and carcinogen on the LDLR expression by CD19 B-cells. Mitogens showed dominance over immunogenic and carcinogenic agents; Bottom, Shows comparison of LDLR expression in different treated groups; Respective p-values between the pairs are shown on the top of the bars.

from more cells attained by cell proliferation. So gene transcription and cell proliferation of B-cells are not parallel events as is clear from this present study. The comparative graphs between different groups (Figure 2A, bottom-panel) have shown that the carcinogenic concentration BaP100 completely inhibits antibody formation and the classical mitogens are immunologically poor agents. A comparison of antibody titer generated



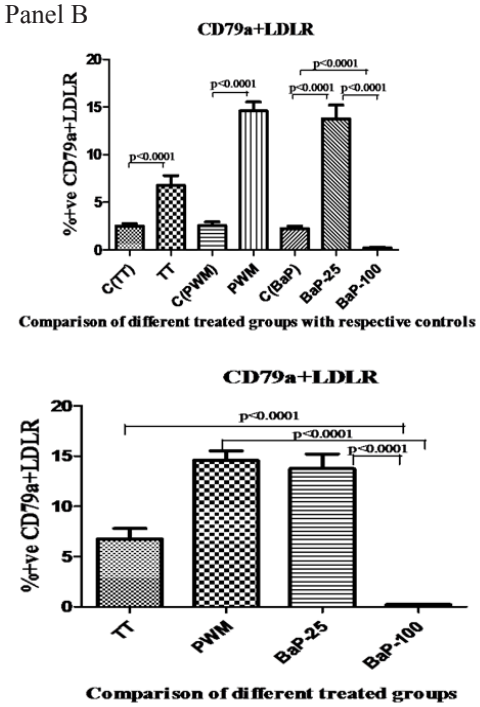
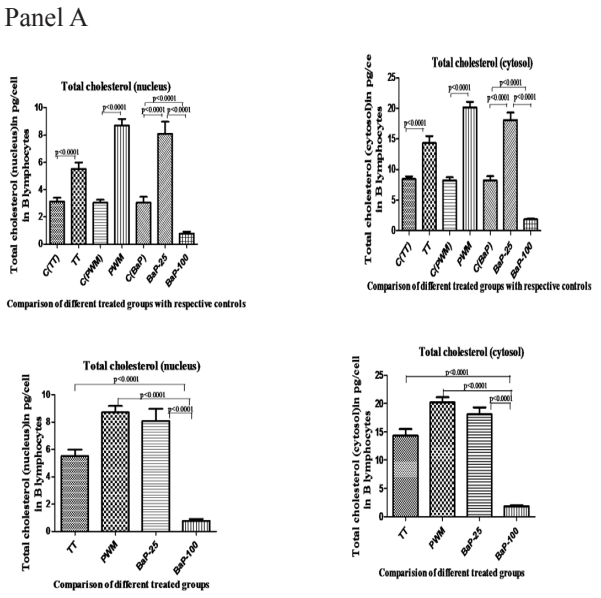


Figure 4. Relative expressions of LDLR on CD79a labeled B-cells by TT, PWM and BaP; Panel-A, Representation of Flow Cytometry for LDLR expression by CD79a B-cells; Panel-B, Top, Bar graphs showing relative potentiality of immunogen, mitogen and carcinogen on the LDLR expression by CD79a B-cells. Mitogens showed dominance over immunogenic and carcinogenic agents; Bottom, Shows comparison of LDLR expression in different treated groups.



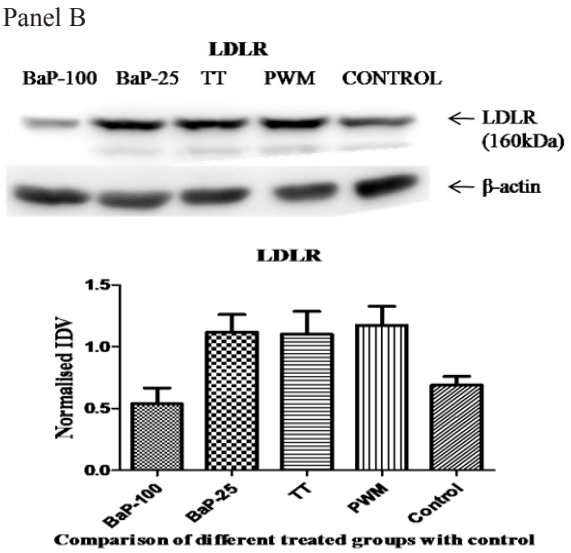


Figure 5. Total Cholesterol Concentration in Cytoplasmic and Nuclear Compartments of Lymphocytes. Panel-A, Top, Shows accumulation of more cholesterol in cell cytoplasm and nucleus by typical mitogens (PWM and Bap-25) as compared to immunogen (TT) or carcinogen (Bap-100); Bottom, Shows comparison of cholesterol concentration among different treated groups; Panel-B, Western Blot on LDLR expression of total splenocytes. TT, PWM and BaP25 have shown comparable effect on LDLR expression while carcinogen (BaP100) lowers the cholesterol concentration in normal lymphocytes. B-actin was used as housekeeping protein marker. Respective p-values between the pairs are shown on the top of the bars.

with similar concentrations of the three inducers is shown (Figure 2B) to represent their relative immunogenic potency.

Intracellular cholesterol pool is maintained by cellular cholesterol synthesis and transport of lipoproteins (Srere PA et al., 1948; Lange Y and Steck TL, 1996; Soccio RE and Breslow JL, 2004; Fabiola B et al., 2015) across surface membrane. Extracellular LDL is the richest source of cholesterol and is transported across plasma cell membrane by LDL-receptor (LDLR). Since the presence of cholesterol in cell nucleus (Albi E and Magni M, 2004; Cascianelli et al., 2008; Albi E et al., 2003; Lajtha A et al., 2007) and its link to cell regeneration is already witnessed (Fernández C et al., 2005; Sun Y et al., 2014; Viola-Magni M and Gahan P,2012; Cascianelli G et al.,2008), the expression of LDLR with B-cell recognition markers has been followed up in this study in presence of TT, PWM and BaP. Relative expressions of LDLR in CD19+

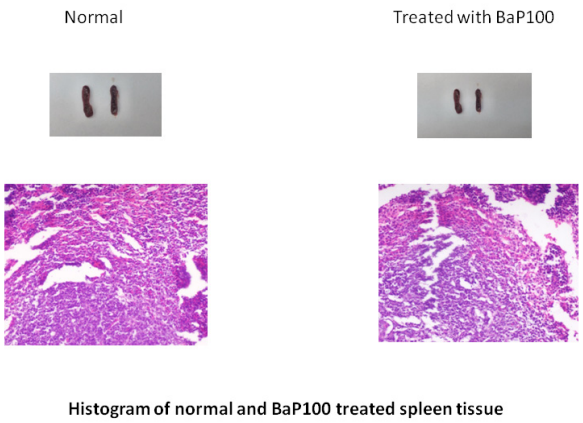


Figure 6. Spleens from Normal and BaP100 Treated Mice. The histogram shows no apparent structural deformity related to the toxicity of carcinogenic dose, BaP100.

cells (B-cells) in presence of the three inducers have been compared in Figure 3. The flowcytometric results for B-cells tagged with fluorescent labeled anti-CD19+ and anti-LDLR in presence of each inducer are shown in Figure 3A. The graphical presentation in Figure 3B shows comparable expression of LDLR between TT, PWM, BaP25 and BaP100. TT shows relatively lower expression whereas with BaP100, LDLR expression is completely turned down. A similar result has been noticed in Figure 4 for LDLR expression in CD79a labeled (other marker for B-cells) cells. This is to mark that the expressions of LDLR on B-cells are in proportion with the cell proliferation profile shown in Figure 1. Rather, the extent of LDLR expressions on BaP100 exposed cells are proportionately much lower as compared to the decreased profile of B-cell numbers (marked with CD19 + CD79a) by BaP100 (shown in Figure 1) against their respective control groups.

To correlate the intracellular cholesterol imported by LDL receptors expressed on cell surface, we have compared the relative mass of cellular cholesterol concentration in cytoplasm and cell nucleus with the extent of LDLR expression in splenocytes isolated after exposures with respective inducers (Figure 5A and B). We have observed a proportional increase of cholesterol concentration in cytoplasm and cell nucleus as with the extent of expression of LDLR in splenocytes (Figure 5B). This shows a direct co-relation of cholesterol transport by LDL receptors remain expressed on cell surface. This is to note that treatment with BaP100 (initial carcinogenic dose) did not show any apparent structural deformity or histological abnormality in tissue sections. Only a slight variation in size was observed with BaP100 which could be because of initiation of cell death or size difference of the mice itself.

Thus this study shows a positive correlation between LDLR expression, intracellular cholesterol concentration and B-cell marker expression prompting an increase in B-cell population with simultaneous increase in LDLR expression and import of cholesterol in cell nucleus through an increase of cholesterol concentration in cell cytoplasm.

Discussion

Potentially all carcinogens are mitogens and shows an uncontrolled mitogenic spark with tumor formation after attaining its cellular level to a critical value called carcinogenic concentration. At this concentration the carcinogens repress tumor suppressor genes, make mutations with epigenetic changes and thus activate oncogenes which then lead the cells to stay indefinitely at exponential growth phase leading to uncontrolled growth and tumourogenesis. It is also known that onset of carcinogenesis associates with immune suppression. But the events behind the immune suppression in the process of carcinogenesis are not yet well understood. We have taken an approach in present study to compare the mitogenic and immunogenic competence of three characteristically reputed components viz TT (immunogen), PWM (mitogen) and BaP (carcinogen). While immune property was verified by estimating the relative profile of generated IgG (antibody) concentrations by the induction of the three agents (TT, PWM, BaP); the mitogenic profile was a function of relative increase in B-cell (CD19+CD79a selected cells) in presence of the same agents. Since the presence of cholesterol is also found in cell nucleus (Albi and Magni, 2004; Cascianelli et al., 2008; Albi et al., 2003; Lajtha et al., 2007) and it has been correlated with tissue regeneration (Fernández et al., 2005; Sun et al., 2014; Viola-Magni and Gahan, 2012; Cascianelli et al., 2008), we intended to see the probability of cholesterol transport within the B-cells with increase of its population and the rating of LDLR expression in CD19 and CD79a positive marker cells. Since it was difficult to follow the fluorescence simultaneously from three entities e.g. anti-LDLR, anti-CD19 and anti-CD79a antibodies in flowcytometry, the expression of LDLR was watched separately in CD19 and CD79a positive B-cells. It is evident from our study that LDLR remained more expressed on PWM and BaP25 exposed cells (Figure 3 and 4) which eventually exploit more mitogenic competence over TT exposed cells (Figure 1). At BaP100, the carcinogenic dose of BaP, only residual expression of LDLR (Figure 3 and 4) was found to exist because of a massive decline of B-cells in presence of BaP100 (Figure 1), although no structural deformity was apparent in the histogram of the BaP100 treated splenocytes (Figure 6). On the contrary, comparatively less LDLR expression has been recorded in TT exposed cells (Figure 3 and 4) showing low profile in B-cell population (Figure 1) but more potential in IgG formation as compared to PWM and BaP as has been evident from Figure 2. By the same reason (decline of B-cells), IgG level for BaP100 exposed cells was too low to count (Figure 2). This is to note that in our recent report (Pandey et al., Online version) we have shown that reduced LDLR expression reduces LDL import into the cells and LDL was found responsible for cell proliferation. Hence low level of LDLR may act as a deterrent of B-cell proliferation or in other words may induce cell death. A comparison of LDLR expression and cholesterol counts in cellular compartments in splenocytes from treated mice separately with three agents has shown more cholesterol in cell cytoplasm and nucleus in PWM

and BaP25 treated cells (Figure 5, panel A and B) as compared to the TT treated counterpart. However, a sturdy absence of cholesterol in cellular compartments of splenocytes in BaP100 (carcinogenic dose) treated cells (Figure 5A) is a reflection of low LDLR expression (Figure 5B) and associated LDL transport which finally induced the cell death (Figure 1).

Therefore, this is apparent from this study that more LDLR expression and more intracellular cholesterol concentration improvise the cells with mitogenic activity which does not mean that those cells will possess more immunogenic property. Immunogenicity is the characteristic property of immune cells and it can be stimulated by characteristic immunogens. As a foreign component the mitogens or carcinogens might have the immunogenic propensity but competently not as high as a classical immunogen (s) e.g. TT. In this study a critical carcinogenic dose has abolished the survival of immune cells and hence abolished the immune shield which could be a protective barrier towards low dose carcinogens or to a mitogen which has not been accumulated at a carcinogenic level. Therefore, in reality, classical mitogens and/or carcinogens are not a typical immunogen, though can be eliminated when present in lower concentration. Only classical immunogens are potentially active in generating substantial antibodies without displaying cell proliferating potency of a typical mitogen. Normal mitogens get destroyed before reaching to their carcinogenic level by allowing proliferation of immune cells within limit to raise an immune barrier against their own mitogenic susceptibility. Carcinogen can expand its carcinogenic activity by abolishing the immune barrier by destroying immune cells, B-cells in this study, and exploiting its mitogenic competence at the exponential level.

In conclusion, the findings in our study show characteristic priority competence of the biological functionary viz. immunogen, mitogen and carcinogen. All these participants may be viewed as potential mitogens but, each one has got priority over its characteristic ingenious property through its mitogenic pluck. In this race, mitogenic excerpts of the critical carcinogenic dose got absolute cell proliferative priority by defeating the immunological shield of the biological system. The level of intracellular cholesterol was found to be a pivotal target within the cells in such occasion.

List of abbreviations

LDLR: Low density lipoprotein receptor; TT: Tetanus Toxoid; PWM: Poke weed mitogen; BaP: Benzo-α-pyrene, PBS: Phosphate buffer saline; PMSF: Phenylmethylsulfonyl fluoride; SDS: Sodium dodecyl sulfate; BSA: Bovine serum albumin; SREBP: Steroid regulatory element binding protein; HRP: Horse radish peroxidase.

Ethics approval and consent to participate

Animal experiments were performed in this study after receiving written consents from the Institute Body for Animal Ethical Clearance (Ref. No: 589/IAEC/11. Date: 17.06.2011).

Consent for publication

All the authors have given their consents for the publication of data generated in this study.

Availability of data and materials

All the data are original and generated in the laboratory of the corresponding author. The animals in this study were obtained from the Institute's animal facility. All chemicals were from reliable sources.

Competing interests

Not applicable.

Funding

This work was supported with funds from the Indian Council of Medical Research (ICMR) Government of India and the Institute Intramural grant, AIIMS, New Delhi India.

The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Nimisha Saxena researched most data, performed the experiments, contributed to discussion and performed statistical analysis wherever needed. Amar Preet Kaur shared experiments for Figure 5. Nimai C. Chandra analyzed all data, contributed to discussion, wrote the manuscript and reviewed/ edited the same in final form.

Authors information

Nimisha Saxena, and Amar Preet Kaur participated in this research work as part of their MD thesis course study. Nimai C. Chandra was acted as a guide and fund raiser for the study.

Acknowledgements

The authors are thankful to Professor D. N. Rao, Department of Biochemistry, AIIMS, New Delhi, India, for providing the facility of animal experiments in his laboratory.

References

Albi E, Cataldi S, Rossi G, Magni MV (2003). A possible role of cholesterol-sphingomyelin/phosphatidylcholine in nuclear matrix during rat liver regeneration. *J Hepatol*, **38**, 623-8.

Albi E, Magni M (2004). The role of intranuclear lipids. *Biol Cell*, **96**, 657-7.

Albi E (2011). Role of intranuclear lipids in health and disease. *Clin Lipidol*, **6**, 59-69.

Balakrishnan K, Adams LE (1995). The role of the lymphocyte in an immune response. *Immunol Invest*, **24**, 233-4.

Bensinger SJ, Bradley MN, Joseph SB, et al (2008). LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell*, **134**, 97–101.

Borena W, Strohmaier S, Lukanova A, et al (2012). Metabolic risk factors and primary liver cancer in a prospective study of 578,700 adults. *Int J Cancer*, **131**, 193.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the

principle of protein-dye binding. *Anal Biochem*, **72**, 248-54.
Bricker LA, Levey GS (1972). Autonomous cholesterol and fatty acid synthesis in hepatomas: Deletion of the adenosine 3′, 5′-cyclic monophosphate control mechanism of normal liver. *Biochem Biophys Res Commun*, **48**, 362-5.
Brown MS, Goldstein JL (1975). Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell*, **6**, 307-6.
Brown MS, Goldstein JL (1986). A receptor-mediated pathway for cholesterol homeostasi. *Science*, **232**, 34-47.
Brown MS, Goldstein JL (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, **89**, 331-40.
Bryan MA, Norris KA (2010). Genetic immunization converts the Trypanosoma cruzi B-Cell mitogen proline racemase to an effective immunogen. *Infect Immun*, **78**, 810-2.
Cascianelli G, Villani M, Tosti M, et al (2008). Lipid microdomains in cell nucleus. *Mol Biol Cell*, **19**, 5289-95.
Chen HW, Heiniger H-J, Kandutsch AA (1975). Relationship between sterol synthesis and DNA synthesis in phytohemagglutinin-stimulated mouse lymphocytes. *Proc Natl Acad Sci U S A*, **72**, 1950-54.
Chen Y, Hughes-Fulford M (2001). Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2. *Int J Cancer*, **91**, 41-5.
Clendening JW, Pandyra A, Boutros PC, et al (2010). Dysregulation of the mevalonate pathway promotes transformation(2010). *Proc Natl Acad Sci U S A*, **107**,15051-6.
Codini M, Cataldi S (2016).Why high cholestrol levels help hematological malignancies: role of nuclear lipid microdomains. *Lipids Health Dis*, **15**, 4.
Cuthbert J, Lipsky P (1990). Mitogenic stimulation alters the regulation of LDL receptor gene expression in human lymphocytes. *J Lipid Res*, **31**, 2067-78.
Dang CV (2012). Links between metabolism and cancer. *Gene Dev*, **26**, 877–90.
Fabiola B, Carlo S, Moschetta A (2015). Uncoupling nuclear receptor LXR and cholesterol metabolism in cancer. *Cell Metab*, **21**, 517 - 26.
Fernández C, Martín M, Gómez-Coronado D, Lasunción MA (2005). Effects of distal cholesterol biosynthesis inhibitors on cell proliferation and cell cycle progression. *J Lipid Res*, **46**, 920-9.
Genaro RV, Jonathan JH, Ciro Z, et al (2016). An LXR-cholesterol axis creates a metabolic co-dependency for brain cancers. *Cancer Cell*, **30**, 683-3.
Gopalakrishnan R, Chandra NC (2006). Estradiol regulates insulin dependent stimulation of LDL-receptor expression in HepG2 cells. *Indian J Clin Biochem*, **21**, 8-14.
Havekes LM, De Wit E, Princen H (1987). Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity. *Biochem J*, **247**, 739-6.
Headley DB, Cohen SM, Bryan GT (1977). Suppression of antibody-mediated and cell-mediated murine immunity by the carcinogen N-[4-(5-Nitro-2-furyl)-2-thiazolyl] acetamide. *Cancer Res*, **37**, 974-9.
Ho Y, Brown S, Bilheimer DW, Goldstein JL (1976). Regulation of low density lipoprotein receptor activity in freshly isolated human lymphocytes. *J Clin Invest*, **58**, 1465.
Janeway CA, Travers P, Walport M, Shlomchik MJ (2001). Immunobiology: the immune system in health and disease. Churchill Livingstone London, chapter 9 and 10.
Jones B (1983). B cell activation by pokeweed mitogen in cultures of normal peripheral blood lymphocytes depleted of T regulator subsets by treatment with OKT4 and OKT8 monoclonal antibodies. *Clin Exp Immunol*, **51**, 461.
Kolomiytseva I, Lakhina A, Markevich L, et al (2016). Fatty acids and cholesterol in the liver cell nuclei of hibernating yakutian ground squirrels. *Dokl Biochem Biophys*, **470**, 364 – 7.
Lajtha A, Galoyan A, Besedovsky H (2008). Handbook of neurochemistry and molecular neurobiology, Neuroimmunology. Springer Science and Business Media. 61-81.
Lange Y, Steck TL (1996). The role of intracellular cholesterol transport in cholesterol homeostasis. *Trends Cell Biol*, **6**, 205-8.
LaVoie HA, Garmey JC, Day RN, Veldhuis JD (1999). Concerted regulation of low density lipoprotein receptor gene expression by follicle-stimulating hormone and insulin-like growth factor I in porcine granulosa cells: promoter activation, messenger ribonucleic acid stability, and sterol feedback 1. *Endocrinology*, **140**, 178-6.
Lindenmann J (1984). Origin of the terms' antibody'and'antigen'. *Scand J Immunol*, **19**, 281-5.
Lo Sasso G, Celli N, Caboni M, et al (2010). Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. *Hepatology*, **51**, 1334–44.
Martínez-botas J, Suárez Y, Ferruelo AJ, Gómez-coronado D, Lasunción MA (1999). Cholesterol starvation decreases P34cdc2 kinase activity and arrests the cell cycle at G2. *FASEB J*, **13**, 1359-70.
Matiasovic J, Leva L, Maskova J, Kummer V, Faldyna M (2008). Effects of postnatal exposure to benzo [a] pyrene on the immunity of immature rats. *Vet Med (Praha)*, **53**, 93.
O'Byrne KJ, Dalgleish A, Browning M, Steward W, Harris A (2000). The relationship between angiogenesis and the immune response in carcinogenesis and the progression of malignant disease. *Eur J Cancer*, **36**, 151-9.
Pahwa SG, Good RA, Hoffmann MK (1981). The role of mitogens and antigens in the generation of antibody-producing human B lymphocytes. *J Clin Immunol*, **1**, 101-5.
Pandey H, Talukdar A, Gangte J, Gupta S, Chandra NC. Cholesterol homeostasis and cell proliferation by mitogenic homologs: Insulin, Benzo-α-pyrene and UV-radiation. Cell Biol Toxicol, https://doi.org/10.1007/s10565-017-9415-8 (Online version)
Rao KN (1986). Regulatory aspects of cholesterol metabolism in cells with different degrees of replication. *Toxicol Pathol*, **14**, 430-7.
S Beloribi-Djefaflia, S Vasseur, F Guillaumond (2016). Lipid metabolic reprogramming in cancer cells. *Oncogenesis*, **5**, e189.
Sanctis D (1998). Expression of low-density lipoprotein receptors in peripheral blood and tonsil B lymphocytes(1998). *Clin Exp Immunol*, **113**, 206-2.
Sankanagoudar S, Singh G, Mahapatra M, Kumar L, Chandra NC (2017). Cholesterol homeostasis in isolated lymphocytes: A differential correlation between male control and chronic lymphocytic leukemia subjects. *Asian Pac J Cancer Prev*, **18**, 23-30.
Saxon A (2004). Immunology: A short course, -by Richard Coico, Geoffrey Sunshine, and Eli Benjamini, Wiley, New York, US $49.95, paper. *Clin Immunol*, **2**, 195.
Singh G, Sankanagoudar S, Dogra P, Chandra NC (2017). Interlink between cholesterol and cell cycle in prostate carcinoma. *Ind J Med Res*, [In press for September 2017 issue].
Singh P, Saxena R, Srinivas G, Pande G, Chattopadhyay A (2013). Cholesterol biosynthesis and homeostasis in regulation of the cell cycle. *PloS One*, **8**, e58833.
Siperstein MD, Fagan VM, Morris HP (1966). Further studies on

the deletion of the cholesterol feedback system in hepatomas. *Cancer Res*, **26**, 7-11.
Skidmore BJ, Chiller JM, Morrison DC, Weigle WO (1975). Immunologic properties of bacterial lipopolysaccharide (LPS): correlation between the mitogenic, adjuvant, and immunogenic activities. *J Immunol*, **114**, 770-5.
Smith J, Osborne T, Goldstein J, Brown M (1990). Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene. *J Biol Chem*, **265**, 2306-10.
Soccio RE, Breslow JL (2004). Intracellular cholesterol transport. *Arterioscler Thromb Vasc Biol*, **24**, 1150-60.
Srere PA, Chaikoff I, Dauben W (1948). The in vitro synthesis of cholesterol from acetate by surviving adrenal cortical tissue. *J Biol Chem*, **176**, 829-3.
Sun Y, Sukumaran P, Varma A, et al (2014). Cholesterol-induced activation of TRPM7 regulates cell proliferation, migration, and viability of human prostate cells(2014). *Biochim Biophys Acta*, **1843**, 1839-50.
Verma A, Chandra NC (2016). Differential Expression of p53, p53R2, hRRM2 and PBR in chronic lymphocytic leukemia: A correlation with intracellular cholesterol. *Indian J Clin Biochem*, **31**, 336 – 1.
Viola-Magni M, Gahan P (2012). Possible roles of nuclear lipids in liver regeneration. *Intech Open,* **4,** 63-78.
Tatidis L, Gruber A, Vitols S (1997). Decreased feedback regulation of low density lipoprotein receptor activity by sterols in leukemic cells from patients with acute myelogenous leukemia. *J Lipid Res*, **38**, 2436-45.
Yang YF, Jan YH, Liu YP, et al (2014). Squalene synthase induces tumor necrosis factor receptor1 enrichment in lipid rafts to promote lung cancer metabolism. *Am J Respir Crit Care Med*, **190**, 675 - 7.
Zalba S, Ten Hagen TL (2017). Cell membrane modulation as adjuvant in cancer therapy. *Cancer Treat Rev*, **52**, 48–57.
Zhenyi An, William A (2016). Weiss. cholesterol: An achilles' heel for glioblastoma. *Cancer Cell*, **30**, 653 – 4.
Zlatkis A, Zak B, Boyle AJ (1953). A new method for the direct determination of serum cholesterol. *J Lab Clin Med*, **41**, 486-2.