Chapter 8

Immunostimulatory effect of Chitosan conjugated green Copper Oxide nanoparticles in tumor immunotherapy

INTRODUCTION:

Metal-based nanoparticles or nano-metalloids are promising tools in several biomedical applications, in particular, in drug and gene delivery (Connor et al., 2005; Pissuwan et al., 2009). These nano-metalloids open a new avenue by acting as a convenient tool for the delivery of antigen to antigen-presenting cell (APC) and nano-vaccine scaffolds (Kostarelos et al.,2007; Kovacsovics-Bankowski and Rock, 1995). As surface chemistry of the engineered nano-metalloids affects the immune response (Bastus et al., 2009), and enhancing the pre-existing adjuvanticity of these nanometalloids remains the main aims (Slutter et al., 2009). These nano-sized particles (NPs) loaded with antigen, freely migrate to lymph nodes and stimulate the immune cells influencing the adaptive immune response (Fifis et al., 2004). Prolonged retention of NPs facilitates antigen uptake by antigen presenting cell (APC) (Melero et al., 2014; Gregory et al., 2013). Another mechanism of the adjuvanticity of the positively charged nano-metalloid NPs is adsorption of negatively charged proteins enhancing their immunogenicity (Deng et al., 2011). Even positively charged chitosan polymers induces inflammasome response (Bueter et al., 2011; Bueter et al., 2014; Fong and Hoemann, 2018) while some other NPs modulate Th1 and Th2 balance (Zhu et al., 2012; Shen et al., 2012).

Cancer immunotherapy is an effective treatment route because in this process natural defense mechanism of body system is boosted up against cancer invasion. Tumor-associated antigens (TAAs) can be recognized by both T cells and B cells; TAAs is the main key regulator of immune reactions. To inhibit tumorigenesis, TAAs targeting using antigens is more common and effective.

Immunomodulating potential of NPs can arise with several side effects which can be neutralized by designing engineered NPs in medicine. Immuno imbalance occurs mainly due to oxidative damage which is responsible for ROS generation to enhance the inflammatory response *in vitro* and *in vivo* model (Liu et al., 2013; Srinivas et al., 2011; Morishige et al., 2012). The shift of Th1/Th2 balance could modulate the immune cells where NF-κB is a key regulator for proinflammatory gene expression by producing cytokines such as TNF-α, IL-1β, IL-6, IL-8 (Zhu et al., 2012; Gao et al., 2011; Wang et al., 2012).

The shape and surface modification of NPs enhances their cell penetration and permeation. This study showed how a spherical, bio-engineered nano-metalloid NPs influences the cellular

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internalization, transport through the receptor and modulation of Th subsets' anti-tumor therapy. In this study, a novel chitosan-coated green copper oxide NPs (CuONPs@CS*) was synthesized and their anti-tumor functions were assessed.

8. MATERIALS:

8.1. Chemicals and Reagents:

Anti $CD4^+$ Ab was purchased from Pertec (Germany Code No-05-8401). Anti IgG, IgG1 and IgG2 were purchased from Imgenex, India. Chitosan, Copper Sulphate, Histopaque 1077, propidium Iodied, were purchased from Sigma (St. Louis, MO, USA). DMEM, RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, sodium carbonate (Na_2CO_3), sucrose and ethylene diamine tetraacetate (EDTA) were purchased from Himedia, India. Tris-Hcl, Tris buffer, KH_2PO_4, K_2HPO_4, alcohol and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Commercially available Pentoxifilline and Sulfasalazine were obtained from Merck Ltd. Dimethyl sulfoxides (DMSO) were procured from Himedia, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd.

8.2. METHODS:

8.2.1. Isolation and preparation of immune cells:

Selection of human subject for lymphocytes isolation was abided by Helsinki protocol. Human peripheral blood mononuclear cells (PBMCs) were collected from nine healthy and normal donors (approved by the Institutional Ethical Committee, Vidyasagar University, Approval No. IEC/6-20(Mod)/C-10/16). After that histopaque 1077 was used to separate the PBMCs from the whole blood by density gradient centrifugation according to Hudson and Hay (1989) (Hudson and Hay 1989). Phytohaemagglutinin (PHA10ng/ml) was used to culture the separated cells. PHA specifically stimulated the T lymphocytes in the lymphocytes culture. Lymphocytes are nonadherent and the macrophages were derived from adherent cells of the culture.

8.2.2. CuONPs@CS incubation with immune cells of our system:

After the separation of Lymphocytes and macrophages ($2x10^5$cells/well), both the immune cells were incubated with CuONPs@CS at a dose of 50µg/ml dose. Incubation was done in a humidified atmosphere (5% CO_2 at 37^oC) for 24hrs (Ponti et al., 2009). Completion of incubation period MTT assay was done to measure cell viability. Following equation was used to calculate the percentage of proliferation.

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% Proliferation = [OD sample – OD control] x 100/OD control

8.2.3. Determination of reduced glutathione (GSH):

From the cell lysate of lymphocytes and macrophages reduced glutathione level was estimated according to Dey et al., 2019. Reduced glutathione levels were expressed as µg of GSH/mg protein.

8.2.4. Determination of oxidized glutathione (GSSG):

The oxidized glutathione level which was expressed as µg of GSSG/mg protein, measured in accordance with the method of Dey et al., 2019.

8.2.5. CuONPs@CS uptake inside the macrophage:

Rh-B tagged CuONPs@CS was up taken by the macrophages which were visualized through the use of a microscope (Dey et al., 2020).

8.2.6. Determination of Anticancer activity of CuONPs@CS pulsed macrophages against MCF-7 and HeLa cells:

Macrophages pulsation with CuONPs@CS was performed with a selected dose of 50µg/ml for 24hrs. Pulsed Macrophages were scraped with scraper and centrifuged at 1500rpm for 5min. Due to centrifugation, macrophages formed a pellet at the bottom of the eppendorf. This pellet was washed with PBS for three times followed by suspended in RPMI 1640 medium with 5% FBS at humidified chamber.

When the cancer cell (MCF-7 and HeLa) culture growth reached the confluence of 80%, the pulsed macrophages were added to it at a ratio of 5:1 and 10:1 (lymphocytes or macrophages**:** cancer cells). The total culture system was incubated for 1, 3 and 5 days at 37^{o}C. MTT assay has been done to measure the viability of cancer cells.

Dead macrophages from the co-culture were discarded by washing with saline. Dead macrophages were easily removed from the well as they tend to float. The live MCF-7 and HeLa cells were assessed by MTT assay. Micro plate reader (model 550, Bio-Rad, Tokyo, Japan) at a wavelength of 570nm was used for measurement of absorbance. The absorbance of control MCF-7 and HeLa cells was consider as 100%. The absorbance of experimental group was divided by the control absorbance (Hino et al., 2005).

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8.2.7. Estimation of cytokines from culture supernatants after pulsation:

According to the manufacturer's instruction by the help of ELISA Kits (BD Biosciences) method lymphocytes and Mφ cytokines (IFN-γ, TNF-α, IL-12 and IL-10) were measured from the supernatant of the cells. In case of lymphocytes and Mφ 50µg/ml dose has been chosen and LPS (1 µg/ml) for 24hr was taken as a reference.

8.2.8. Incubation with TNF-α inhibitor and NF-κβ inhibitor:

Pentoxifylline (POF) was added into the macrophage culture at a concentration of 2mM for 2hrs. Then macrophages were incubated with LPS and CuONPs@CS for 24hrs. After incubation macrophages became pulsed. These pulsed macrophages were co-cultured with MCF-7 and HeLa cells at a 10:1 ratio of E (Effector cells): T (Target cells) for again 5 days. After incubation period cytotoxicity was calculated by the MTT assay. Similarly Sulfasalazine (SAZZ) was added into the macrophage culture at concentrations of 2mM. Both POF and SAAZ were added at concentration of 2mM each to assess their synergistic effect in the cultivated macrophage (Dey et al., 2020).

8.2.9. Flow cytometric analysis of $CD4^+$ population after pulsation with CuONPs@CS:

NPs (50µg/ml of CuONPs@CS) pulsed macrophages were co-culture with total PBMCs and incubated for 24hrs. The incubation time period was 30mins at room temperature with Pacific Blue A conjugated anti-human $CD4^+$ monoclonal antibody. Therefore the PBMCs were washed and again suspended in PBS and analysed with a FACS CANTO II flow cytometer (BD Biosciences).

8.2.10. Preparation of lysate antigen and conjugation with CuONPs@CS:

Cancer cell suspension was centrifuged and the pellets were suspended in cold PBS. The concentration of cells will be $2x10^5$cells/ml maintained 4 cycles of freezing and thawing process. Then lysates were centrifuged to remove the debris and stored at -20^{o}C according to Prasad et al., 2011. The conjugation of the lysate antigen with the nanoparticles was performed according to Solbrig et al., 2007.

8.2.11. Generation of Cytotoxic T lymphocytes and anti-cancer assay:

The NPs pulsed macrophages were co-cultured with the isolated T cells. T cells isolation was performed by nylon wool column. In another group the MCF-7 and HeLa cell extract was added

with CuONPs@CS and then co-cultured with T cells. At a ratio of 1:10 of target cells: effector was added in all groups. The target cells were MCF-7 and HeLa and effector cells were T cells. Co-culture was performed in 96-well micro titer plates at 37^{o}C with T cells and CuONPs@CS pulsed macrophages. All the groups were incubated for 5days. MTT was done by the above mentioned process subsequently after 5 days of completion of incubation (Dash et al., 2015).

8.2.12. Immunization of Animal:

Pertaining to guidelines of National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, six-week aged female Balb/c mice were used. Maintenance of mice were approved by the Ethical Committee of Vidyasagar University (approval no IEC/6-10(Mod)/C-9/16). One week time was taken by the mice to adapt. Then mice were immunized using a reference dose as per Cho et al., 2012. Immunization process was done at 2-week intervals. According to Cho et al., 2012, tail vain injection was injected with a dose of 100µl dose containing 25µl PBS, 50µl BSA (100µg) and 25µl CuONPs@CS solution (50µg). PBS solution was used as a negative control. Following the 2nd sensitization on the 7th day, blood was obtained from the tail vein. Subsequently after 7 more days, second intraperitoneal blood stimulated by antigen was obtained through cardiac vein puncher method. Finally the Serum from the blood was processed for immunoglobulin (IgG) estimation.

8.2.13. Assessment of tumor growth restriction assay:

The total female Balb/c mice were divided into three groups. Each group contained 5 mice. Groups were I) Unpulsed Mφ

II) CuONPs@CS pulsed Mφ

III) CuONPs@CS + cancer cell lysate pulsed Mφ

Immunization was done three times in a week. After completion of the immunization, 3 days later 4T1 tumor cells were inoculated (1×10^{7}) subcutaneously in the abdominal mammary gland (Goswami et al, 2010). After 4T1 cells inoculation, the tumor weight was measured at an interval of 3days (after 4T1 inoculation) up to 21 days. During this period tumor size was measured. The % of survivability was estimated by Kapler meiyer method.

8.2.14. Estimation of Protein:

Protein estimation was performed according to the method of Lowry et al., 1951.

8.2.15. Statistical Analysis by Origin 6.1:

Entire data were represented as mean ± SEM. Means of two groups were analogized using two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with several comparison t-tests. $p<0.05$ was taken as a limit of significance**.**

8.3. RESULTS:

8.3.1. Selection of biologically safe dose and redox balance:

Lymphocytes and macrophages are the primary immune cells of system. The CS coated green CuONPs (CuONPs@CS; 1-100µg/ml) toxicity on these immune cells was studied by MTT assay. 50µg/ml dose was selected due to minimal toxicity against macrophage and lymphocytes

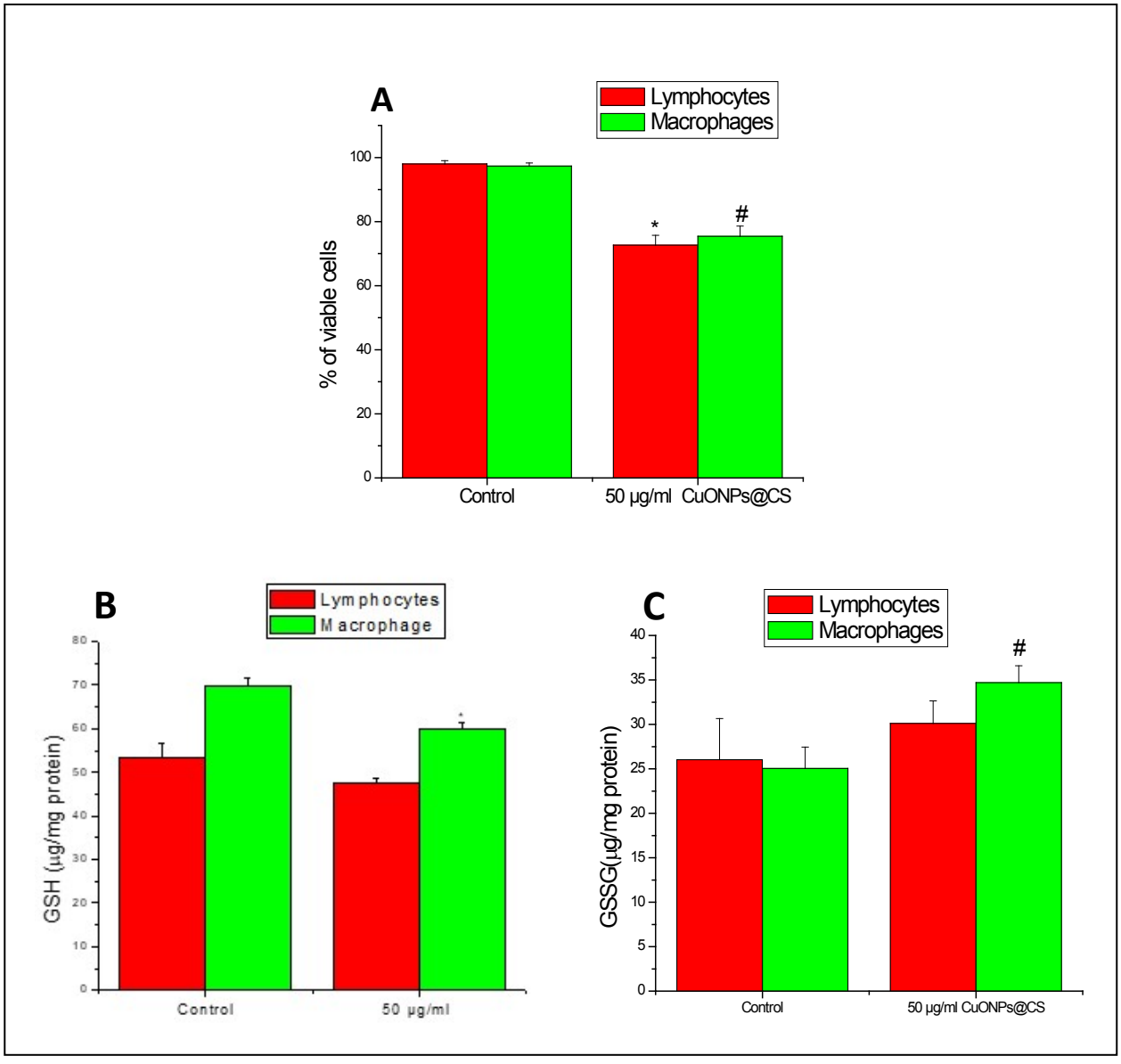

Figure 8.1: **A)** Cytotoxicity of CuONPs@CS against Macrophage and lymphocytes at a particular dose of 50µg/ml for 24hr. **B)** Measurement of GSH and **C)** GSSG of macrophage and lymphocytes after the application of CuONPs@CS. Values are expressed as mean ± SEM of three experiments; *superscripts indicate significant difference ($p<0.05$) compared with the lymphocytes control group. #superscripts indicate significant difference ($p<0.05$) compared with the macrophages control group.

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from the previous chapter. But green synthesized CuONPs at 50µg/ml dose, killed 55.57% of lymphocytes and 48.55% of macrophages which showed significant ($p<0.05$) toxicity. GSH level at a 50µg/ml dose did not produce significant toxicity in comparison with the control ($p<0.05$) of lymphocytes and macrophages (Fig. 8.1).

8.3.2. Uptake of CuONPs@CS by macrophages:

Successful internalization of CuONPs@CS (50µg/ml) inside the MΦ was observed under fluorescence microscopy after tagging the NPs with Rh-B (Fig. 8.2 A, B & C).

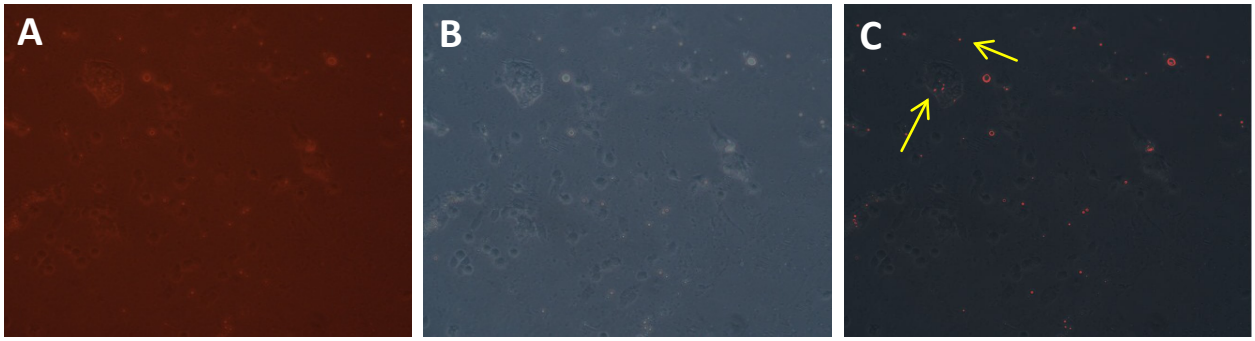

Figure 8.2: A) Fluorescence image of Internalization of Rh-B tagged CuONPs@CS inside the macrophage. **B)** Grey scale image of Internalization of Rh-B tagged CuONPs@CS inside the macrophage **C)** Overlay image of Internalization of Rh-B tagged CuONPs@CS inside the macrophage.

8.3.3. Anticancer activity by CuONPs@CS pulsed macrophages:

CuONPs@CS pulsed macrophages were co-cultured with cancer cells at ratios of 5:1 and 10:1 (MΦ: cancer cell). This co-culture was maintained for 1, 3 and 5 days. After 5 days, cell viability was significantly reduced in both the treated cancer cell lines with CuONPs@CS triggered macrophages. Both ratios were effective but 10:1 ratio significantly depleted the % of viable cells ($P<0.05$), in comparison with 5:1 ratio and the untreated control cells. At a ratio of 10:1, CuONPs@CS pulsed macrophage after co-cultured with cancer cells; viability of MCF-7 cell was 81.31%, 64.42% and 58.42% after day 1, 3 and 5 (Fig. 8.3B). But in case of HeLa cells, viability was 76.24% (after day 1), 62.88 % (after day 3) and 45.73% (after day 5) (Fig.8.3D). Green synthesized CuONPs showed more cancer cell viability than the CuONPs@CS at a dose of 50µg/ml after the activation of macrophages. The CS coated green CuONPs were able to activate the macrophages but without coating green CuONPs showed toxicity towards immune cells which was already observed from previous study (Dey et al., 2019).

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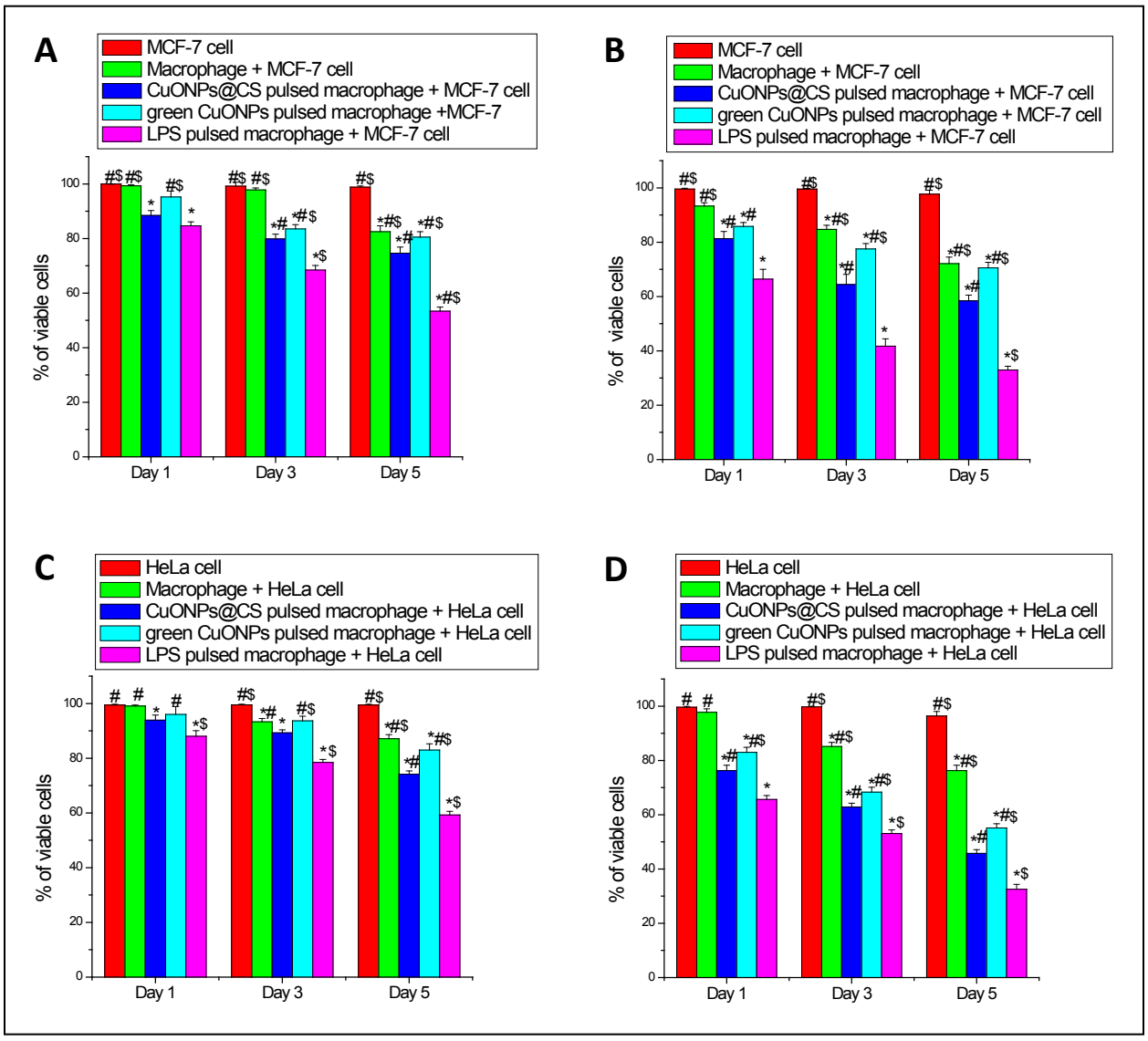

Figure 8.3: A) .Cytotoxicity assay of CuONPs@CS and LPS pulsed macrophage co-culture with MCF-7 cells at a) 5:1 **B)** 10: 1 and HeLa cells at **C)** 5:1 and **D)** 10:1. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant difference ($p < 0.05$) compared with the control group that is only MCF-7 or HeLa cell. $^{\#}$superscripts indicate significant difference ($p < 0.05$) compared with the Mφ + cancer cells. $^{\$}$superscripts indicate significant difference ($p < 0.05$) compared with the LPS pulsed Mφ + cancer cells.

8.3.4. Cytokine release assay:

Lymphocytes and macrophages treated with the nano-metalloid showed 2.68 fold and 3.22 fold increased in TNF-α production, respectively compared to the control group but green synthesized CuONPs treated lymphocytes showed 3.71 fold and macrophages showed 3.77 fold higher level of TNF-α compared to the control. However IFN-γ production was increased by 2.12 fold and

by 3.96 fold, respectively, IL-12 production was augmented by 2.24 folds and 3.93 folds, respectively; but IL-10 production was reduced by 1.99 folds and 1.52 folds, respectively compared to the control group (Fig. 8.4). In case of all the entire Pro-inflammatory cytokines levels increased after the treatment with only green synthesized CuONPs compared to the CuONPs@CS. Surface coating helped to reduce the Pro-inflammatory cytokines level.

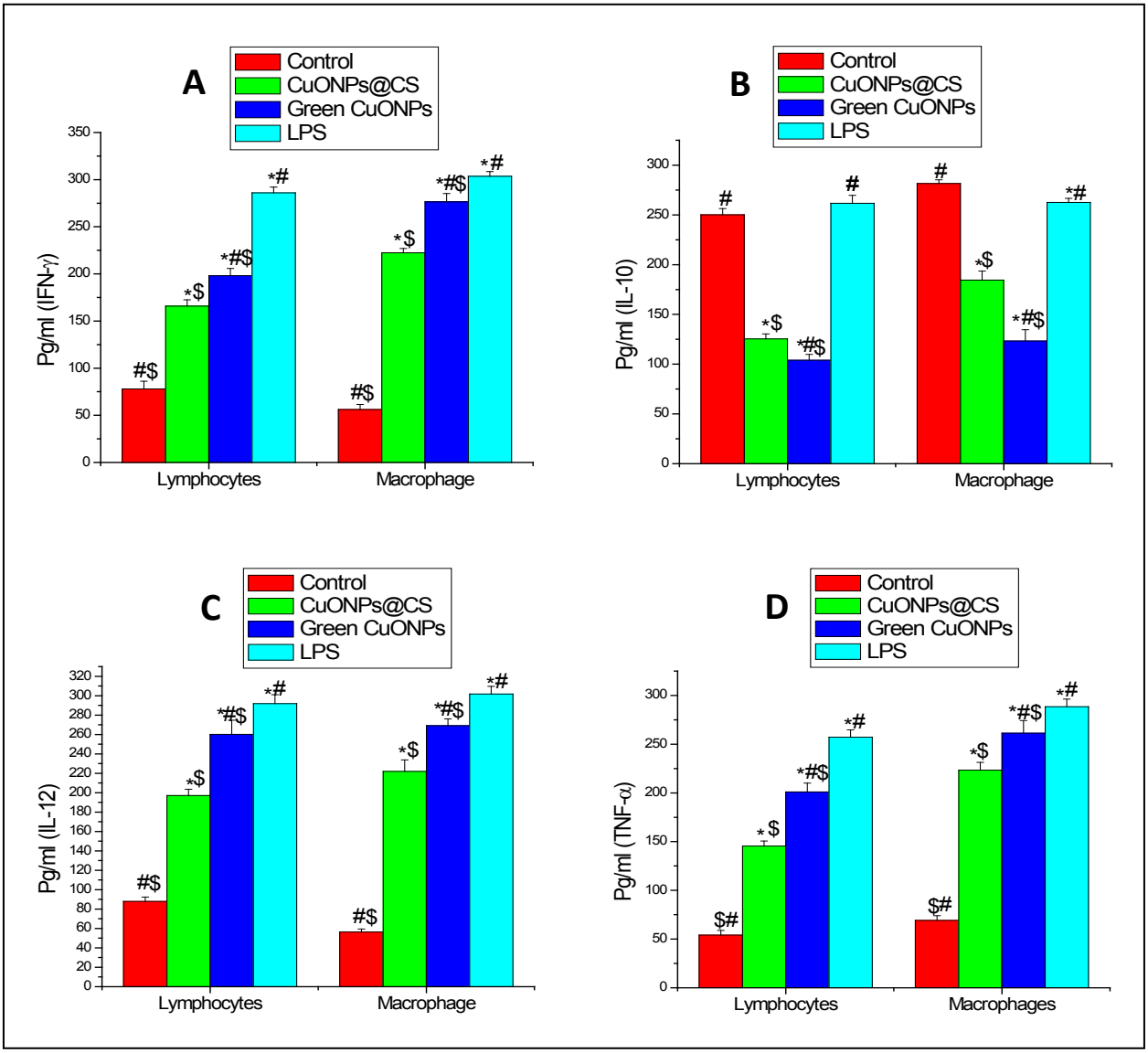

Figure 8.4: Assessment of cytokine released from CuONPs@CS pulsed Macrophage. **A)** IFN-γ, **B)** IL-10, **C)** IL-12 and **D)** TNF-α. Values are expressed as mean ± SEM of three experiments; *superscripts indicate significant difference ($p<0.05$) compared with the control group. #superscripts indicate significant difference ($p<0.05$) compared with CuONP@CS treated group. $superscripts indicate significant difference ($p<0.05$) compared with LPS treated group

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8.3.5. Incubation with POF and SAAZ:

When cAMP accumulates in the cells, Pentoxifylline which is an inhibitor of TNF-α, suppressed the macrophage to produce TNF-α. Increment of cancer cell (MCF-7 and HeLa) viability was observed by using POF in the culture media. MCF-7 viability increased up to 86.24% and HeLa viability increased up to 72.95% (Fig.8. 5A).

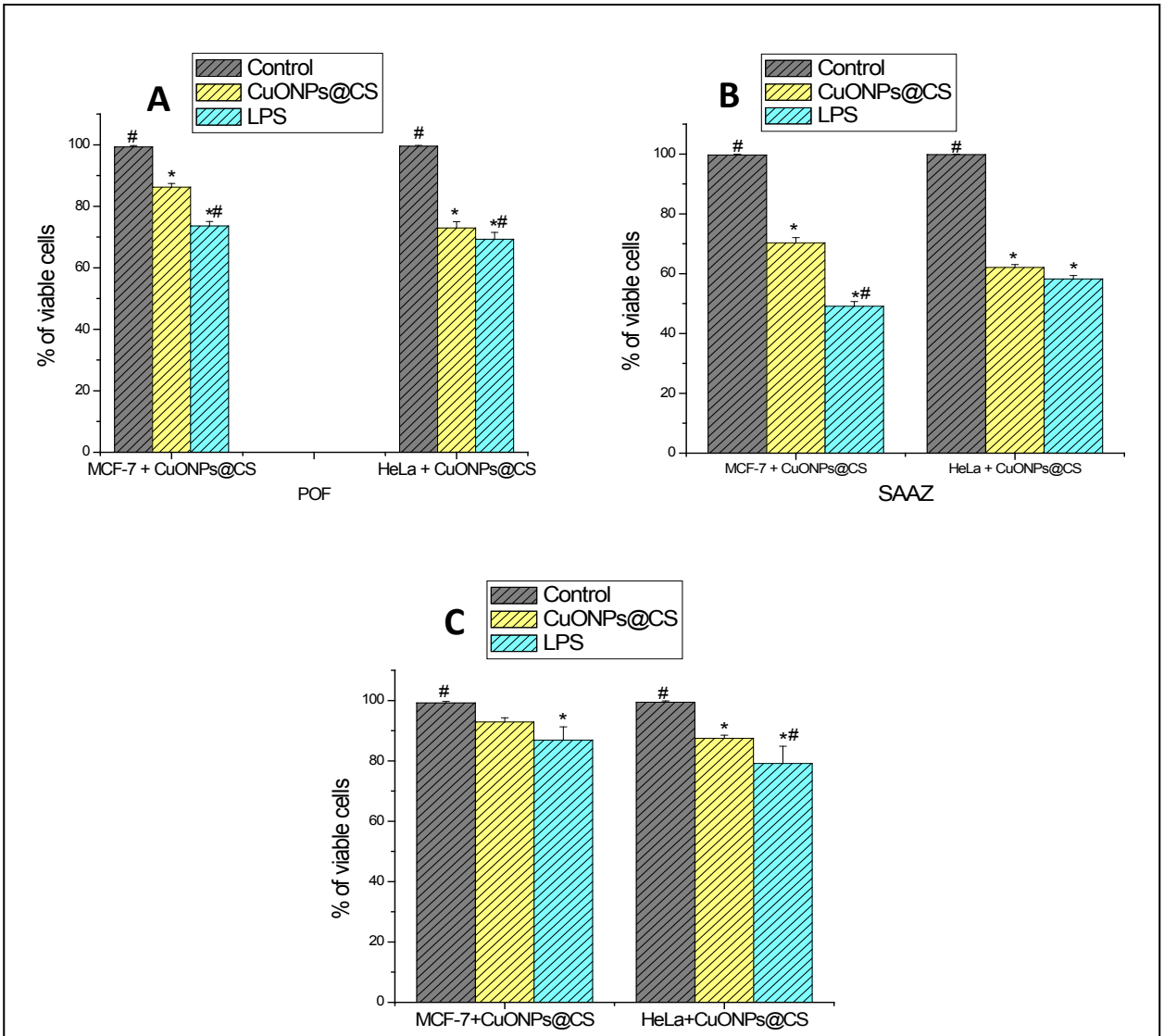

Figure 8.5: Estimation of viability of cancer cells after co-culture with CuONPs@CS pulsed macrophage at a 1:10 ratio in the presence of inhibitors **A)** POF, **B)** SAAZ and **C)** POF and SAAZ together. Values are expressed as mean ± SEM of three experiments; *superscripts indicate significant difference ($p < 0.05$) compared with the control group. #superscripts indicate significant difference ($p < 0.05$) compared with the CuONPs@CS treated group.

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After blocking with only SAAZ, the viability of MCF-7 increased up to 70.32% and HeLa up to 62.06% (Fig. 8.5B). After blocking with two inhibitors (POF & SAAZ) together it was observed that the viability of both cancer cells increased significantly. MCF-7 viability increased up to 92.88% and HeLa up to 87.47% (Fig.8.5 C).

8.3.6. Effect of CuONPs@CS on $CD4^+$ lymphocyte population:

$CD4^+$ lymphocytes population was increased after the treatment with CuONPs@CS pulsed macrophages. When CuONPs@CS pulsed macrophages were co-cultured with PBMCs for 48hrs $CD4^+$ cells population amplified significantly in this group (Fig.8.6A- 8.6I) in comparison with negative control group. In case of CuONPs@CS pulsed macrophages group showed $CD4^+$ cell population near about positive control (LPS treated macrophages) of the experiment which was very significant phenomenon in this study. The negative control group was only macrophages without any treatment.

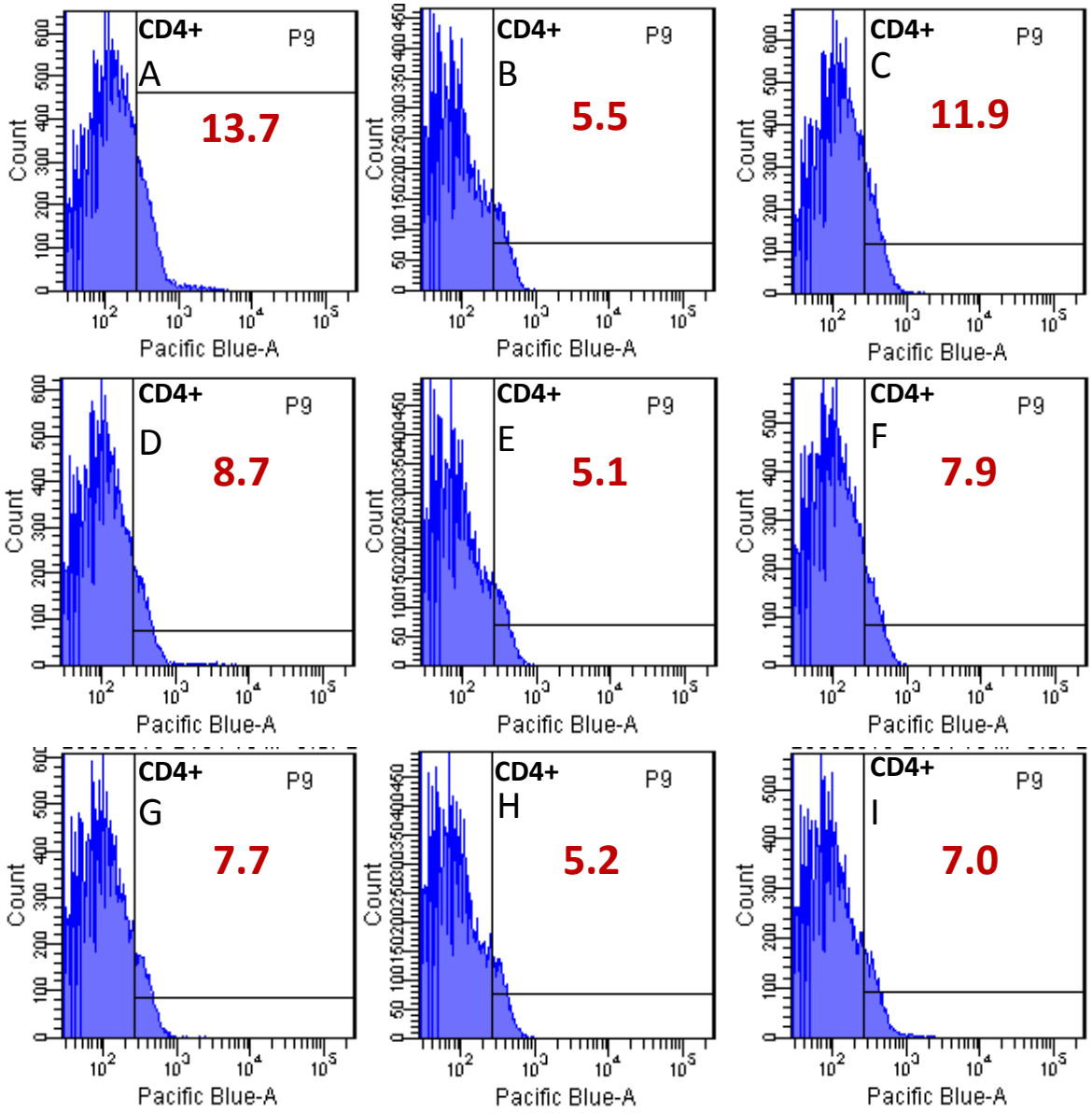

Figure 8.6: CD4+ population was measured by FACS after co-culture with CuONPs@CS pulsed macrophage at a 1:10 ratio. **(A, D, G)** Positive Control, **(B, E, H)** Negative control, **(C, F, I)** CuONPs@CS treated.

8.3.7. Assessment of T cell mediated cytotoxicity:

Macrophages were pulsed with MCF-7 and HeLa cancer cell lysate and CuONPs@CS. Then the pulsed macrophages were co-cultured with T cell population for 48hrs at a ratio of 1:10. The activated T cells produced CTL against specific cancer antigen. The targeted cancer cells were neutralized by the antigen specific generated CTL. The % of cytotoxicity was measured by MTT assay. Percentage of cytotoxicity was higher in case of cancer cell antigen + CuONPs@CS pulsed macrophage group (Fig. 8.7).

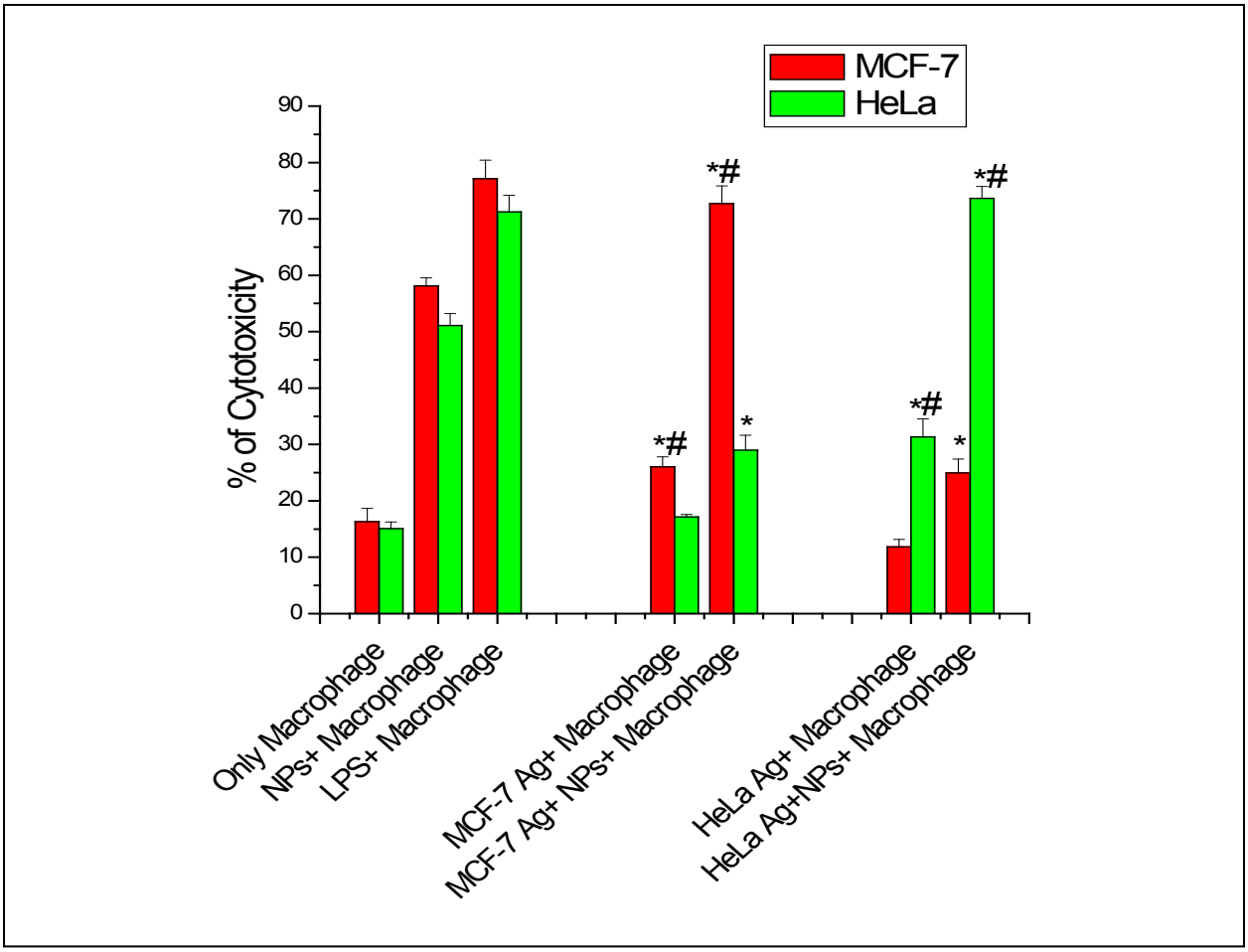

Figure 8.7: Cytotoxicity of MCF-7 and HeLa cells after co-culture with cytotoxic T lymphocytes at a 1:10 ratio. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant difference ($p<0.05$) compared with the only macrophage group. #superscripts indicate significant difference ($p<0.05$) compared with the NPs (CuONPs@CS) + Macrophages.

8.3.8. *In vivo* study:

8.3.8.1. Estimation of Immunoglobulin:

After the administration of 200µl of PBS-BSA-CuONPs@CS, the serum LDH level increased slightly compared to the only PBS treated group indicating no significant toxicity by PBS-BSA-CuONPs@CS. Anti-BSA IgG concentration increased after the administration of CuONPs@CS. CuONPs@CS produced elevated level of IgG1 and IgG2 response (Fig. 8.8 C &D).

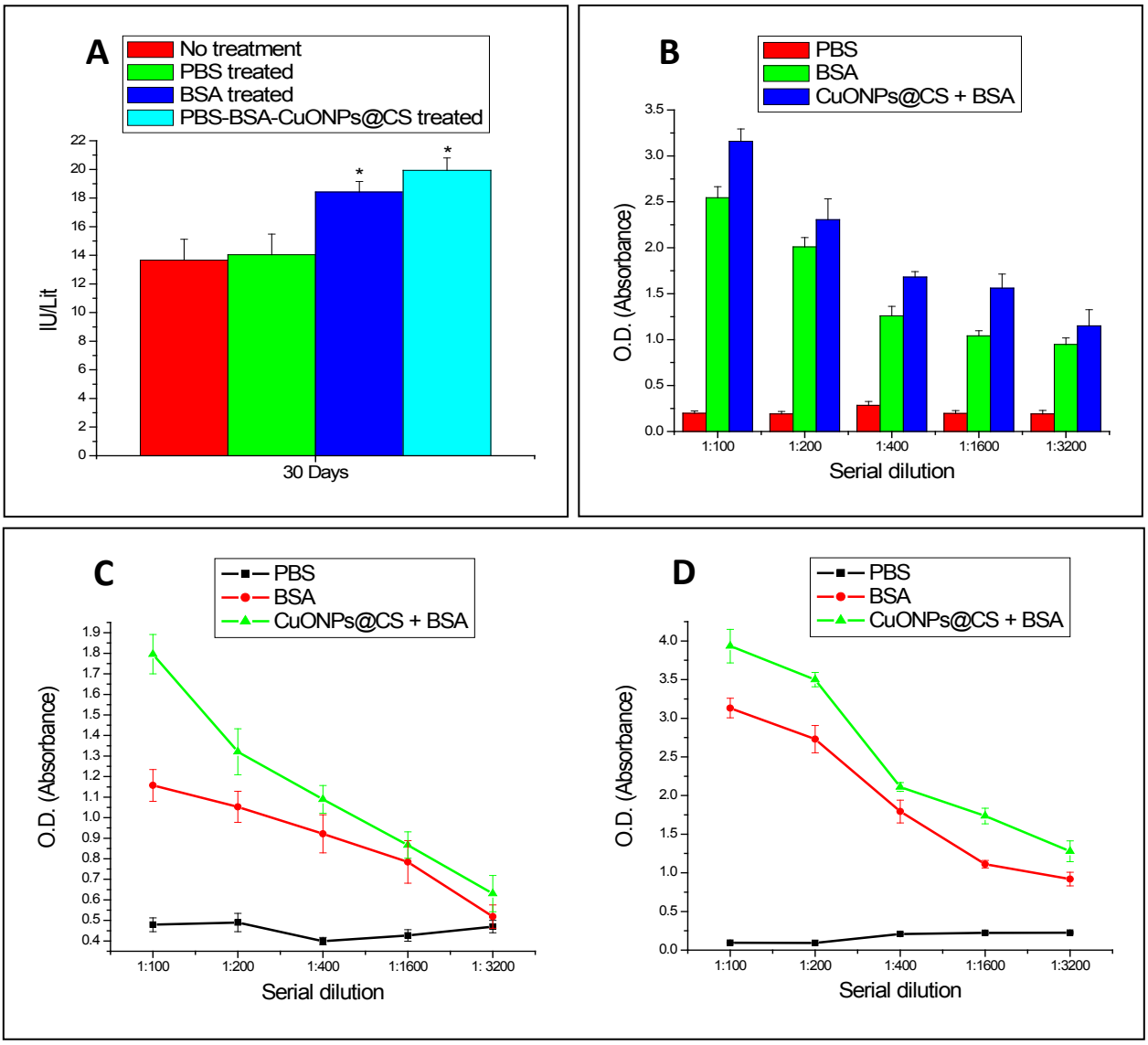

Figure 8.8: A) Estimation of LDH from mice serum **B)** Colorimetric estimation of anti BSA IgG level in serum after second subcutaneous sensitization at an interval of 3 days. **C)** Estimation of BSA specific IgG1 and **D)** IgG2 level at day 7. Values are expressed as mean ± SEM of three experiments; *superscripts indicate significant difference ($p < 0.05$) compared with the control group.

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8.3.8.2. Splenocyte Proliferation assay:

BSA and CuONPs@CS conjugated particles influenced the spleen cell population which was observed through FACS analysis (Fig. 8.9 A & B) compared to the control group PBS treated spleen cells.

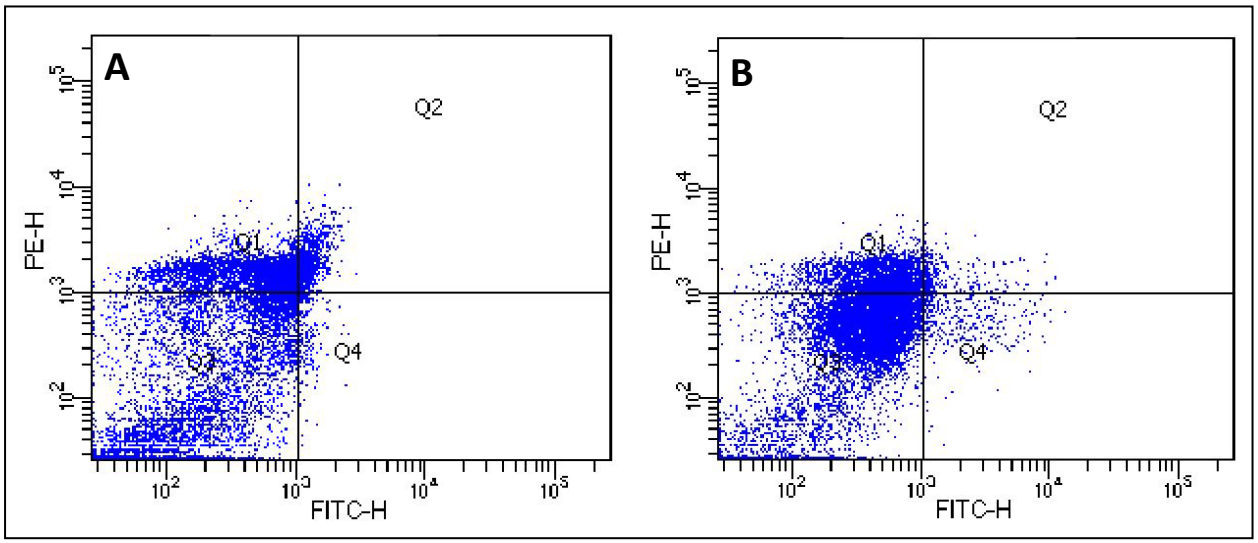

Figure 8.9: Spleen cell population by FACS analysis **A)** Spleen cell of only PBS treated group **B)** Spleen cell of CuONPs@CS treated group.

8.3.8.3. Tumor growth restriction assay and survivability:

The treatment with the CuONPs@CS conjugate prolonged the survival of tumor-bearing mice. Balb/c mice were 40% survivability rate and CuONPs@CS conjugate with antigen lysate group showed 60% survivability rate (Fig. 8.10). The enhanced survival was accompanied by significantly reduced tumor weight (1.6g in conjugate treated from 3.5g in control after 21 days; $p<0.05$) (Fig. 8.11).

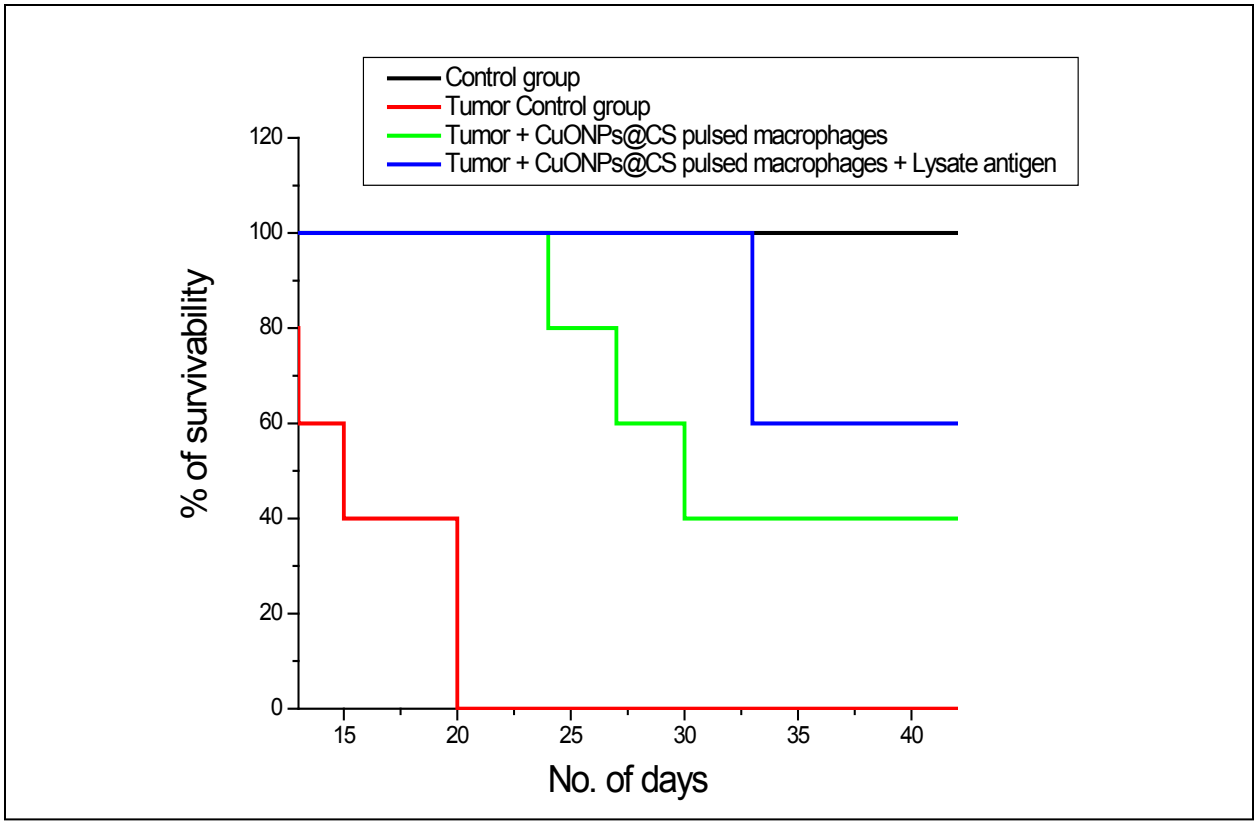

Figure 8.10: Estimation of survivability of Tumor bearing mice after the immunization with different groups for 3 times in a week until day 21. n=5.

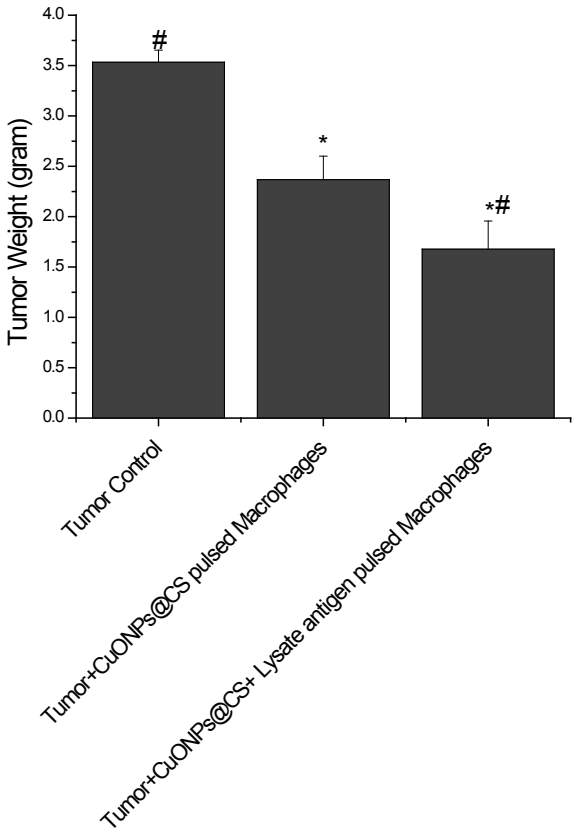

Figure 8.11: Reduction in tumor weight after immunization of mice with different groups for 3 times in a week until day 21. Values are expressed as mean±SEM; *superscripts indicate significant difference ($p<0.05$) compared with the control group. #superscripts indicate significant difference ($p<0.05$) compared with the Tumor+ CuONPs@CS pulsed Mφ group. n=5.

8.3.9. DISCUSSION:

Nanoparticles are able to produce oxidative stress and to induce inflammation as reflected in the production of several cytokines (Naqvi et al., 2010; Liu et al., 2013; Srinivas et al., 2011). NPs with unique physico-chemical property can modulate the tumor microenvironment by the macrophage activation that may constitute an effective way for cancer immunotherapy. In this

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present study, it observed that the spherical CuONPs@CS NPs of about 34.5±5nm diameter activated macrophages and executed efficient anti-tumor immunity perhaps due to cationic property of the CS polymer. Immune response depends on the tailoring chemistry of the surface of the NPs (Moon et al., 2012; Elsabahy and Wooley, 2013). In the previous study, it was observed that no significant cytotoxicity against lymphocytes at a dose of 50µg/ml after coating the NPs with CS. Simultaneously CS helped to release more Cu ions in the acidic environment (Sadigh-Eteghad et al., 2013; Ye et al., 2014) but the p^H of lymphocytes and macrophages were almost neutral at which very little amount of Cu ions released. Although multi-layer coating may helped to reduce the ion dissolution rate from the core metal NPs that might helped to reduce toxicity. CuONPs@CS pulsed macrophages killed breast and cervical cancer cells significantly ($P<0.05$) compared to the control group; LPS that triggered strong inflammatory responses, was a positive control (Juskewitch et al., 2012). Pulsed macrophage was co-cultured with cancer cells at a ratio of 10:1. After day 5, both cancer cells were killed significantly ($P<0.05$) compared to the control. Several Th1 cytokines (TNF-α, IFN-γ and IL-12) were produced from the macrophage which indicated the activation of macrophage. Increment of Pro-inflammatory cytokines (TNF-α, IFN-γ and IL-12) level and depletion of anti-inflammatory IL-10 level was observed in the study. Overproduction of IFN-γ and IL-12 made a positive feedback loop to trigger Th1 response whereas Th2 cytokines are inactivated by IFN-γ (Hsieh et al., 1993). IL-10 which was secreted by Th2 cells produced several types of cytokines. Among these cytokines IL-10 immediately after the secretion suppressed the cytokine IL-12, IFN-γ which are produced by Th1 cells (Liu et al., 2016).

To evaluate the mechanistic pathway of cancer cell apoptosis, two inhibitors of TNF-α and IFN-γ were used. It was found that after blocking the TNF-α and NF-κβ the cancer cell viability increased which ensured the involvement of both proteins in cancer cell apoptosis and TNF-α binds to TNF-RI receptor of TNF-α and activating members of the caspase family (Soares et al., 1998). CuONPs@CS pulsed macrophage increased the proliferation of CD+4 T cell. These activated Th1 cell influenced various cytokine by which it can regulate the immune system. The effects of IFN- γ on Th1 development may be mediated via activated macrophage which up-regulated the IL-12 production (Trinchieri, G. 1995). Th1 cells released IFN-γ which activated macrophage and cytotoxic T cell, indicated cell mediated immunity. In the CTL response assay (Fig. 8.7) it was observed that specific cancer cell antigen with NPs, pulsed macrophage and

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generated Cytotoxic T cell Lymphocytes against Ag positive cancer cells significantly. Possibly the synthesized NPs conjugate with cancer cell antigen activated the DCs (dendritic cells) cell those are responsible for robust CTL response. The conjugate served as an antigen delivery vehicle. MHC molecules represent antigens towards T cells. MHCI molecules were identified through CD8+ T cells which was produced by cytotoxic T lymphocytes reaction whereas MHCII molecules are recognized by CD4+ T cells (induced Th1 cells) (Heegaard et al., 2010; Khan and Reddy, 2013). In this context it was clearly demonstrated that CD4+ and CD8+ T lymphocytes are activated due to Ag (antigen) specific CuONPs@CS triggered macrophage. CuONPs@CS was able to produce anti BSA IgG in the serum by the production of Th1 and Th2 cell and confirmed by spleen cell proliferation (Fig. 8.9B). This result confirmed the adjuvant role of CuONPs@CS which induced IgG response in mice sera. CuONPs@CS with BSA produced significant amount of IgG1and IgG2 in mice model. This result indicated that BSA antigen specific T cell response was generated and during this response CuONPs@CS acts as an adjuvant. Similar kind of result was found by Desmedt et al., 1998. This phenomenon indicated that potent adjuvancity role of CuONPs@CS which inhibited the cancer cell proliferation by using specific lysate antigen in union with NPs. Th1 dominance was reflected in the high value of IgG2 which influenced humoral immunity. Th1 cells regulated the inflammatory response. Th2 cells influenced B cells differentiation and produced immunoglobulin IgG which regulated humoral immunity (Elsabahy and Wooley, 2013). This study puts the emphasis on the immunostimulant role of the conjugate through the cellular and humoral immunity. IgG response in the sera indicated the activation of both Th1 and Th2 cells. The conjugated nanoparticle activated the NADPH via ROS generation and subsequently activated the macrophage which helped in the elevation of pro-inflammatory cytokines by modulating the tumor microenvironment. At the same time, antigen associated conjugated NPs showed (Fig. 8.7 and 8.11) better anticancer activity due to macrophage activation implicating that the NPs may act as an antigen delivery vehicle in the system. IgG in the serum of mice indicated the activation of both Th1 and Th2 cells.

Generally adjuvants act as immune modulators. Adjuvant associated with antigen drive immune response from innate to adaptive. The nano conjugate can deliver the adjuvant associated with specific antigen can activate the macrophages. The CuONPs@CS boosted up the immune response by acting as an adjuvant.

The present study clearly demonstrated that cancer cell lysate antigen with CuONPs@CS successfully killed the MCF-7 and HeLa cells selectively *in vitro* and *in vivo*. It helped to reduce the solid tumor burden and upsurged the % of survivability of the tumor-treated mice. TNF-α activated the macrophage strongly and inhibited the cancer cell proliferation as well as produced antibody dependent and antibody-independent cytotoxicity which allowed the long time survival of macrophage (Eriks and Emerson, 1997; Biedermann et al., 2004).

CS conjugated green CuONPs induced Th1 cells by producing several cytokines that activated the macrophages to eradicate the cancer cells. Alteration in redox status deciphered the ROS generation which interplay between depleted level of GSH and intracellular signaling or co-stimulatory molecules, i.e., adjuvant (CuONPs@CS). The immunostimulatory role of CuONPs@CS was improved and simultaneously evaluated its anti-tumerogenic response via instigation of CD4+ T cells. An effective immunotherapeutic tool that can confer anticancer activity in *in vitro* and *in vivo* system was designed.

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