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Differential levels of CD20 play crucial role in response of antibody mediated cell death in Burkitt's Lymphoma Cell Line 'Daudi'

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Abstract

To assessed the sensitivity of human Burkitt's lymphoma cells to rituximab and tositumomab at differential levels of CD20 molecules on cell surface. Cells that possess low CD20 levels exposed with γ -radiation and thereafter treated with mAbs. During present investigation we found that cells treated post irradiation showed higher levels of cell death. Moreover, tositumomab found to be strong cell death inducer. In addition, Bax / Bcl-2 ratio were also found higher in associated with differential levels of CD20 expressed cells as well as significant inhibition of pro-survival signaling such NF- κ B and Akt pathways. These findings demonstrated that the efficacy of anti-CD20 mAbs is dependent on the surface levels of CD20. Based on these results, we hypothesized that irradiation just prior to immunotherapy may provide new treatment options even in aggressive B-cell lymphoma, which are having low levels CD20 or even resistant to current therapies *in vivo*.

Key-Words: Antibody, CD 20, Daudi

Introduction

Cancer remains is a global concern worldwide. It has emerged as the second leading cause of death globally after cardiovascular diseases. The International Agency for Research on Cancer (IARC) recently estimated that 7.6 million deaths worldwide were due to cancer with 12.7 million new cases per year being reported worldwide [1]. In India, deaths from the disease have increased by 60% according to the latest report 'Global Burden of Cancer-2013', released worldwide on Friday (May 29, 2015). Among them non-hodgkin lymphoma is the most common type of cancer in the world. Approximately 71,850 new cases and 19,790 deaths were reported due to non-hodgkin lymphoma in 2015 (Surveillance, Epidemiology and End Results Program 2015).

Rituximab was the first FDA (US) approved anti-CD20 monoclonal antibody used for relapsed and refractory indolent lymphoma against the B-cell specific human CD20 cells surface molecules [2]. To date several anti-CD20 mAbs such as rituximab, ^{90}Y -Ibritumomab, tositumomab, ofatumumab and Obinutuzumab (GA 101) etc. have got FDA approval for use in NHLs, RA and others are in various stages of development.

In clinical applications, the efficacy of anti-CD20 mAbs seems to be decline after a period of months, leading to create therapeutic resistance. The explanation for this therapeutic resistance is not clear. Although, some investigators provide information in relation to decreased level of CD20 expression and/ or harbor low levels of CD20 on surface of malignant B-cells is one of the major contributing factor for antibody response [3, 4]. However, there is general agreement that diseases such as chronic lymphocytic leukemia may display the CD20 cell surface molecule in fairly low titer and may respond proportionally less well to rituximab and other mAbs as compared to other low grade B-cell malignancies [4-7]. CD20 a non-glycosylated transmembrane protein, exclusively expressed on B-cells. It appears during the pre-B cell stage, however absent during the earlier or later stages of B-cell differentiation such as antibody secreting plasma cells [8, 9]. The binding of anti-CD20 mAbs with CD20 induced the activation of members of the *src* family of tyrosine kinases, elevation in intracellular Ca^{2+} , phospholipase C γ activation are collectively leads to apoptosis[10, 11]. Apoptosis is a tightly regulated process of cell death, which is triggered by caspase dependent or independent manner and controlled by the interplay of a number of positive and negative regulatory proteins such as Bcl-2 and Bax family

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members [12-16]. Moreover, anti-CD20 mAbs are also able to induce inhibition of major pro-survival pathways such as ERK1/2 MAPK, nuclear factor κ B (NF- κ B) and AKT, which directly or indirectly regulated pro-apoptotic and anti-apoptotic machinery [17, 18].

Thus, the changes in CD20 levels on malignant B-cells would be a new treatment option for cancer prevention. Philippe et al provide first information in relation to radiation induced changes in CD20 expression on B-cells [19]. Later on, Kunala et al was also suggested that exposure of ionizing radiation (10Gy) can increase CD20 surface expression in a dose and time dependent manner in IM9, IM9/Bcl-2 and Ramos neoplastic B-cell lines. Gupta et al strongly suggested that the significant increase in cell-surface expression of CD20 was transient and cell-type dependent manner in logarithmically growing Daudi and Raji cells [20]. Recently, Singh et al demonstrated that sub-lethal dose (0.5Gy) of γ -radiation can induce ~3 fold CD20 levels on Burkitt's lymphoma cell line 'Daudi' and it was associated with changes in oxidative condition in intracellular milieu [21, 22].

Based on these problems we hypothesized that cells treated with anti-CD20 mAbs just after exposure of sub lethal dose of γ -radiation may be improve antibody mediated cell death. During present investigation, we found more antibody response rate at higher levels of CD20 expression. Moreover, Bax/Bcl-2 ratio was also found to be higher as well as we additionally found significant inhibition of pro-survival pathways such NF- κ B and Akt.

Material and Methods

RPMI-1640, Penicillin G potassium salt, streptomycin, N-2-Hydroxyethyl piperazine N-2-Ethane sulphonic acid sodium (HEPES sodium), Dimethyl sulfoxide (DMSO), sodium pyruvate, Bradford reagent, Amifostine, Protease and Phosphatase inhibitor Cocktail were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS), 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA), 3, 3'-dihexyloxycarbocyanine iodide (DiOC₆) (3), Propidium Iodide (PI) was obtained from Invitrogen (USA). Anti-CD20 chimeric antibodies, such as rituximab (Rtx) were obtained from Genentech (Genentech, Inc., South San Francisco, CA), and tositumomab (Tst) from Corixa (Corixa Corporation Seattle, WA). QuantiBRITE beads and anti-CD20-PE was obtained from BD bioscience (USA). All other chemical used were of AR grade and obtained from local manufacturers from SRL and Himedia India. Cell culture and biochemical purpose related plastic wares

were obtained from BD bioscience (USA), Corning (USA) and Tarsons, INDIA.

Cell culture

Daudi cells was obtained from the American type culture collection, (USA) and culture was maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 10mM HEPES, 50units/ml penicillin, 50 μ g/ml streptomycin, and 1% non-essential amino acids at 37°C in a humid atmosphere with 95% air and 5% CO₂.

Irradiation and treatment of cells

Exponentially growing cells were exposed with γ -radiation, 0.5Gy and 1.5Gy separately using ⁶⁰Co-teletherapy unit (Bhabhatron-II Telecobalt unit; obtained from Bhabha Atomic Research Center (BARC), Mumbai, India) at a dose rate of 1.67Gy/min. Control groups were treated similarly except for irradiation. After irradiation cells were incubated at 37°C in a humid atmosphere with 95% air & 5% CO₂ for further experiments.

Ionizing radiation (IR) induced changes in expression of CD20 was measured at different time intervals. Cells exposed to low doses of IR were further treated with anti-CD20 mAbs (5 μ g/ml, Tst and Rtx respectively for all experiments) to measure antibody response at differential levels of CD20 on cell surface. Individual sham irradiated controls and antibodies alone were taken separately.

CD20 expression

The changes in levels of CD20 on the surface of cells were measured flowcytometrically using anti-CD20 antibodies conjugated with PE at an Ex λ 488nm and Em λ 578nm[20-22]. The fluorescence was also acquired using quantiBRITE beads and numbers of CD20 molecules on the surface of cells (CD20 molecules/cells) were calculated from standard curve obtained from fluorescence of beads.

Redox regulation of CD20 cell-surface levels

The redox regulation of CD20 levels on cell-surface followed low dose γ -radiation were measured as previously described method [20]. Amifostine have strong antioxidant properties is known to neutralize free radicals intermediates. Cells (5 \times 10⁵ cells/ml in complete RPMI) were pre-treated with a low concentration of amifostine (0.1 μ g/ml, 1 h, 37°C), followed by irradiation (0.5Gy), and thereafter cells were washed (to remove antioxidants) and incubated in complete RPMI 1640 at 37°C. Cell-surface expression of CD20 was measured by flow cytometry at different time intervals. Briefly, after treatment, cells were washed with ice-cold PBS, blocked in FACS buffer (0.5% BSA, 1% heat-inactivated human serum, and 0.1% sodium azide in PBS, pH 7.2), and incubated

with anti-CD20 PE-conjugated antibody. After a 15-min incubation at 4°C (in the dark), 10,000 events were acquired using a FACS can flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using Flow Jo 7.6.5 software (Tree Star, Inc., San Carlos, CA, USA). The results were expressed as fold change in CD20 expression.

Measurement of anti-CD20 mAbs mediated cell death

Anti-CD20 mAbs induced cell death at differential levels of CD20 expression on cell surface followed by low dose γ - radiation was measured as PI uptake by flowcytometrically[20-22]. PI is membrane impermeable dye, generally excluded from viable cells and therefore, commonly used for identifying dead cells in a population. Briefly, cells were harvested and incubated with PI (5 μ g/ml) at room temperature in the dark to determine death cells. After 5min incubation in dark, 10,000 events/ sample were acquired at Ex λ 488 \pm 10nm and Em λ 617 \pm 10nm using FACS can flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analysed using Flow Jo 7.6.5 software (Tree Star, Inc., San Carlos, CA, USA). The results are expressed as fold induction cell death from mean fluorescence \pm SD of three independent experiments.

Protein isolation and estimation

Briefly, samples were centrifuged and cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1% Nonidat-P40, 5mM EDTA, 1mM EGTA, 1mM PMSF, protease and phosphatase inhibitor cocktails) using vigorously vortexing. Samples were centrifuged at 10,000 rpm and carefully supernatant containing soluble proteins was transferred in fresh vials. An aliquot of each sample was used to measure protein concentration by the Bradford method [23] using bovine serum albumin (BSA) as standard. The remaining sample was mixed with 4x SDS sample buffer (1x equals 31.25mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, and 0.005% bromophenol blue) and heated for 5 min at 95°C.

Western blotting

To study the changes in expression of Bax/Bcl-2 proteins and pro-survival signaling pathways were measured as previously described method [20-22, 24, 25]. Proteins concentration was measured by Bradford method in each sample and equal amount of proteins (60 μ g/ sample) of different samples were loaded in 12% SDS poly-acrylamide gel. A standard molecular weight protein marker (Bio-Rad, USA) was loaded in in separate well parallels with sample. Initially, 40 volts of current was supplied for formation of protein stack. Thereafter, constantly run at 100 volts and

proteins were resolved in denaturing 12% bis-acrylamide separating gel. After complete separation of proteins, proteins were transferred from SDS poly-acrylamide gel on to a nitrocellulose membrane. The membranes were blocked in blocking buffer (3% BSA prepared in tris buffer saline with 0.1% Tween-20 for determine phosphorylated proteins and/ or 5% skimmed milk prepared in tris buffer saline with 0.1% Tween-20 for determination of non-phospho proteins) for 1hrs at room temperature and overnight at 4°C. Thereafter, membranes were incubated for overnight at 4°C with desired primary antibody *viz.* anti-NF- κ B; 1:1500, anti-Akt; 1:1000, anti-Bcl-2; 1:2000 and anti-Bax; 1:1500. After incubation, membrane were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 4hr at room temperature. Thereafter, membranes were washed and protein bands were visualized by using enhanced ECL chemiluminescence Pico. β - Actin was used to ensure equal loading of protein samples. Band intensity was determined using GS-900™ Calibrated Densitometer software. The results were expressed as fold changes from mean of densitometer data \pm SD of three independent experiments.

Data analyses and statistical evaluations

Assays were set up in triplicates and the results were expressed as the mean \pm SD. The significant changes in between group were analyzed by student's t-test and *p* values were shown at different levels of significance (**p*<0.05, ***p*<0.01, ****p*<0.001).

Results and Discussion

Changes in CD20 levels

The increase in CD20 molecules on cell surface was the major aim of the present study for treatment of resistance and low level CD20 expressing Burkitt's lymphoma cells. The kinetics of single dose of γ - radiation (0.5Gy) induced alterations the levels of CD20 molecules on cell surface was measured using flowcytometry at different time points (ranging from 0-36hrs) (Figure 1A and B). The overlay of index histograms is clearly showing significant changes in CD20 expression (Figure 1A). The surface levels of CD20 on cell surface was also found to be maximum at 20hrs (3.8 \pm 0.1 Lakh/cell) post irradiation and remained higher till 36hrs (Figure 1B).

Redox regulation of CD20 expression

Exposure of cells to radiation is known to induce intracellular generation of free radicals cause oxidative stress. To determine the role of intracellular redox changes or oxidative stress in the expression of CD20, cells were treated with known ROS scavenging molecule such as amifostine just 1hr before irradiation. The results of γ -radiation induced changes in the

expression of CD20 on cell surface also correlated with the ROS levels at all-time points in Daudi cells. Cells treated with amifostine (WR-2721; FDA approved pro-drug) 1hr pre-exposure with 0.5Gy radiation were used to measure both ROS levels and expression of CD20 using flowcytometry at 8hr, 20hr and 36hr. The results were showed significant inhibition in levels of both ROS and CD20 expression simultaneously in all-time points studied (Figure 2A and B). Remarkably, amifostine is not involved in changes CD20 expression. Amifostine (WR-2721) converts into pharmacologically active metabolite WR-1065 upon de-phosphorylation having free radical scavenging potential and stability.

Anti-CD20 mAbs mediated cell death

The ligation of anti-CD20 mAbs with CD20 induced multiple cell death process such as antibody dependent cellular cytotoxicity (ADCC), ROS induced cell death; lysosomes mediated and direct induced programmed cell death *in vitro*. An apparent cell death was measured flowcytometrically using PI uptake (Figure 3). As shown in Figure 3PI positive population were counted and represent as histograms. The fold changes in cell death were determined from mean fluorescence values. The cells were exposed with 1.5Gy radiation or treated with Tst were showed significant level of cell death (sham irradiated control vs 1.5Gy irradiated; ### $p < 0.001$, control vs Tst treated; \$\$\$ $p < 0.001$ respectively). The cells treated with Rtx alone or in combination with radiation (0.5Gy+Rtx and 1.5Gy+Rtx, 20hr post irradiation) showed 2.5 ± 0.4 and 4.5 ± 0.2 increase fold cell death ($***p < 0.001$) respectively as compared to Rtx alone (Table 1). Whereas, cells treated with Tst alone or their combination with 0.5Gy radiation (0.5Gy+Tsts; 20hr post irradiation) showed 1.5 ± 0.2 fold increase in cell death ($**p < 0.05$) and with 1.5Gy radiation (1.5Gy+Tst; 20hr post irradiation) showed 5.7 ± 0.3 fold increase in cell death ($***p < 0.001$) compare to Tst alone (Table 1). Moreover, cells treated with corresponding isotype antibodies showed no cell death (Table 1).

Preparation of protein sample

Equal amount of protein sample were used for immunoblotting to determine precise changes in expression of genes or proteins levels. The preparation of sample for western blotting proteins concentration in each samples were measured using Bradford method and concentration determined using BSA standard curve. The proteins concentration in each sample were determined using bovine serum albumin (BSA) standard as $1\text{OD} = 8.5\mu\text{g protein}$.

Western blotting

Despite the success role of differential levels of CD20 on cell surface in anti-CD20 mAbs induced cell death *in vitro* in the Burkitt's lymphoma cell line 'Daudi'. Further, we were investigated the anti-CD20 mAbs induced attenuated the expression of pro-apoptotic and anti-apoptotic gene products such as Bax and Bcl-2 respectively in association with changes CD20 levels. As shown in figure 4A the significant up-regulation of Bax expression in case of cells exposed to 0.5Gy or 1.5Gy and in cells treated either Rtx or Tst with or without 1.5Gy radiation. The up-regulation of Bax levels was observed just 2 ± 0.3 fold in cells exposed with 0.5Gy and 7 ± 0.2 fold increase Bax levels was observed in 1.5Gy irradiated cells as compared to sham irradiated control. Moreover 3 ± 0.3 fold increase Bax expression was determined in 0.5Gy irradiated cells and 5 ± 0.2 fold observed in 1.5Gy exposed cells. However the significant increase in Bax expression was also observed in cells treated with Rtx or Tst at 20hr post radiation exposure (0.5Gy, 1.5Gy). Approximately 1.5 fold was observed in 1.5Gy+Rtx group and 2.3 ± 0.2 fold in 1.5Gy+Tst group as compared to Rtx and Tst respectively.

Besides the measurement of the up-regulation of Bax expression, the inhibition of Bcl-2 expression was also observed. The inhibition of Bcl-2 expression was found to be significantly higher as dose dependent manner and also in treatment of cells with anti-CD20 mAbs alone or in combination of anti-CD20 mAbs + IR (Figure 4B). The cells exposed with 0.5Gy or 1.5Gy and thereafter measuring the Bcl-2 expression was found to be inhibited as 40% and 58% as compared to sham irradiated control respectively. In addition cells treated with Rtx or Tst alone in separately were found significant inhibition if Bcl-2 expression compared to control ($$$$p < 0.001$). However cells treated with Rtx or Tst at 20hr post 1.5Gy radiation were also found to be significantly ($*p < 0.05$) as compared to Rtx or Tst treated alone. Our above observations revealed that treatment of cells with either Rtx or Tst preferentially inhibited the expression of the anti-apoptotic gene product Bcl-2 and beside up-regulated the expression of pro-apoptotic gene product Bax level.

We were further investigated whether Rtx or Tst inhibited signaling pathways that regulate these gene products. We were found that Rtx and Tst have ability to down-modulate the nuclear factor- κ B (NF- κ B) and AKT survival pathways which participate in down-regulation of Bcl-2 expression in Burkitt's lymphoma cell line 'Daudi' (Figure 5A and B). NF- κ B is a nuclear transcription factor that plays important role in immunity, cellular survival and apoptosis through the

regulation of genetic networks. During present investigation the changes in expression of RelA (p65) was determined followed by Rtx and Tst treatment or combination with IR (Figure 5A). Our observation revealed that cells treated with Rtx and Tst separately showed significant inhibition in expression of p65 ($p < 0.001$) compared to control. Interestingly cells treated Rtx or Tst at 20hr post irradiation showed higher levels inhibition in p65 expression compared to control. However combination of Rtx+IR or Tst+IR was also showed significant inhibition of p56 expression compared to Rtx or Tst alone ($*p < 0.05$). Beside the changes in expression of p65, inhibition in expression of AKT was also found to be significant compared to untreated control (Figure 5B).

CD20 a non-glycosylated transmembrane protein, exclusively expressed on transformed B-cells [8, 9]. It has received extensive evaluation as an ideal target for immunotherapy and radio-immunotherapy, in part because of its ubiquitous expression, stable localization within the cell membrane of target cells (transformed B-cells) [26]. The ligation of CD20 with mAbs induced various cell death processes such as complement-dependent cytotoxicity (CDC) antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC) and can directly induce programmed cell death (PCD) [27-30]. Thus, the ability to selectively control CD20 expression may provide new therapeutic approach for NHLs, specifically those which carrying low levels of CD20 antigens on cells surface or reduce CD20 surface levels on cell.

During present studied we assumed that, radiation-induced changes of CD20 surface levels may play a crucial and central role in determining the relative efficacy of rituximab and tositumomab in treating Burkitt's lymphoma disease. Here, we examined the γ -radiation-induced modulation of CD20 levels on surface of the Burkitt's lymphoma cell line 'Daudi'. Cells exposed with sub-lethal dose of γ -radiation (0.5Gy) showed significantly increased in CD20 expression as time dependent manner and found to be maximum at 20hr (3.8 ± 0.1 Lakh/ cell) and remained higher till 36hr and thereafter it reached to near sham irradiated control (2 ± 0.2 Lakh/ cell) with increase of time (Figure 1A and B). The results are also correlated with our previous findings related to changes in CD20 expression followed by radiation dose and time dependent manner [31]. Further we studied the regulation of changes CD20 expression followed by radiation exposure. In correlation to previous findings, treatment of cells with amifostine (WR-2721) thereafter exposure of cells with 0.5Gy γ -radiation

showed significant inhibition of CD20 levels on cell surface as well as decrease levels of ROS (Figure 2A and B). Amifostine (WR-2721) is an FDA approved phosphothiol prodrug, which forms the pharmacologically active metabolite WR-1065 upon dephosphorylation [32, 33]. WR1065 is known for its free radical scavenging potential and stability. WR-1065 enters the cell predominantly by passive diffusion or *via* the polyamine transport system [34]. The dephosphorylation of WR-2721 may initiate the thiol/disulphide exchange reaction and thereby influences intracellular thiols *viz.* cysteine and GSH [35, 36]. Upon entry of WR-1065 into the cells, it naturalized the potential of redox modifiers *viz.* ROS and RNS. Based on the previous and present study it concluded that ROS and RNS may be responsible for inhibiting the radiation-or ROS mediated expression of CD20. Moreover, to determine relative efficacy of anti-CD20 mAbs at differential levels of CD20 expression, cells treated with rituximab and tositumomab alone and 20hr post radiation exposure. It is well known that anti-CD20 mAbs can activate classical Fc-dependent mechanisms such as ADCC, ADCP, and CDC under *in vivo* condition [27-29]. However, certain anti-CD20 mAbs can also eliminate B cells by triggering intracellular signaling on ligation with antigen and directly induce programmed cell death (PCD) *via* cross-linking/ homotypic adhesions (aggregations) and hyper-crosslinking [29, 30, 37, 38]. During present investigation we found significant cell death in combination of radiation and mAbs as compared in respect to sham control (Figure 3). Interestingly, tositumomab was found potent induce of cell death as compared to rituximab (Table 1).

In addition, we were also investigated the ratio of Bax and Bcl-2 followed by treatment of radiation and mAbs. The members of the Bcl-2 family are a group of crucial regulatory factors in apoptosis. We studied the protein expression of the pro-apoptotic members such as Bax and the anti-apoptotic member such as Bcl-2 after induction of anti-CD20 mAbs mediated apoptosis. The permeabilization of mitochondrial outer membrane is regulated by Bcl-2 family proteins and it can bind selectively to the active conformation of Bax to prevent it from inserting into the mitochondrial outer membrane to maintain the normal permeability of membrane permeable transition pores (MPTPs), and prevent the release of mitochondrial pro-apoptotic factors, such as cytochrome c, AIF and Smac/DIABLO [39-42]. Here we found significant inhibition in expression of Bcl-2 and up regulation of Bax (Figure 4A and B). Moreover, anti-CD20 mAbs are also able to inhibition of major pro-survival pathways such as ERK1/2 MAPK, nuclear

factor κ B (NF- κ B) and AKT [17, 18] which directly or indirectly regulated pro-apoptotic and anti-apoptotic machinery. Thus, we were further investigated whether rituximab or tositumomab inhibited signaling pathways that regulate these gene products. Cells treated with both rituximab and tositumomab separately showed significant inhibition in expression of two major cell survival pathways viz NF- κ B and Akt (Figure 5A and B) survival pathways which participate in down-regulation of Bcl-2 Burkitt's lymphoma cell line 'Daudi'.

Conclusion

Present studied suggest that increase in cell surface CD20 expression is associated with increase cell death induced by anti-CD20 mAbs. In conclusion, this report provides evidence that CD20 expression can be induced by low dose γ -radiation. Therefore, use of irradiation just prior to immunotherapy may be beneficial for eradication of B cell malignancy. These results may be useful to establish a theoretical basis to improve the efficacy of immunotherapy/ radio-immunotherapy in case of cells expresses low surface levels of CD20.

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Author Contributions

Conceived and designed the experiments: Vijay Singh and Damodar Gupta. Performed the experiments: Vijay Singh. Analyzed the data: Vijay Singh, Damodar Gupta. Contributed reagents/ materials/ analysis tools: Damodar Gupta, Wrote the paper: Vijay Singh and Damodar Gupta.

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Table 1: Anti-CD20 mAbs mediated cell death at differential levels of CD20 on cell surface

Sample	Fold Changes	SD
Control	1	0.275006
0.5Gy	1.69837	0.332885
1.5Gy	3.707881	0.365777
Rtx	1.690218	0.251888
0.5Gy+Rtx	3.800272	0.356282
1.5Gy+Rtx	5.914403	0.261559
Tst	3.029892	0.444892
0.5Gy+Tst	4.764946	0.301485
1.5Gy+Tst	8.538045	0.195849

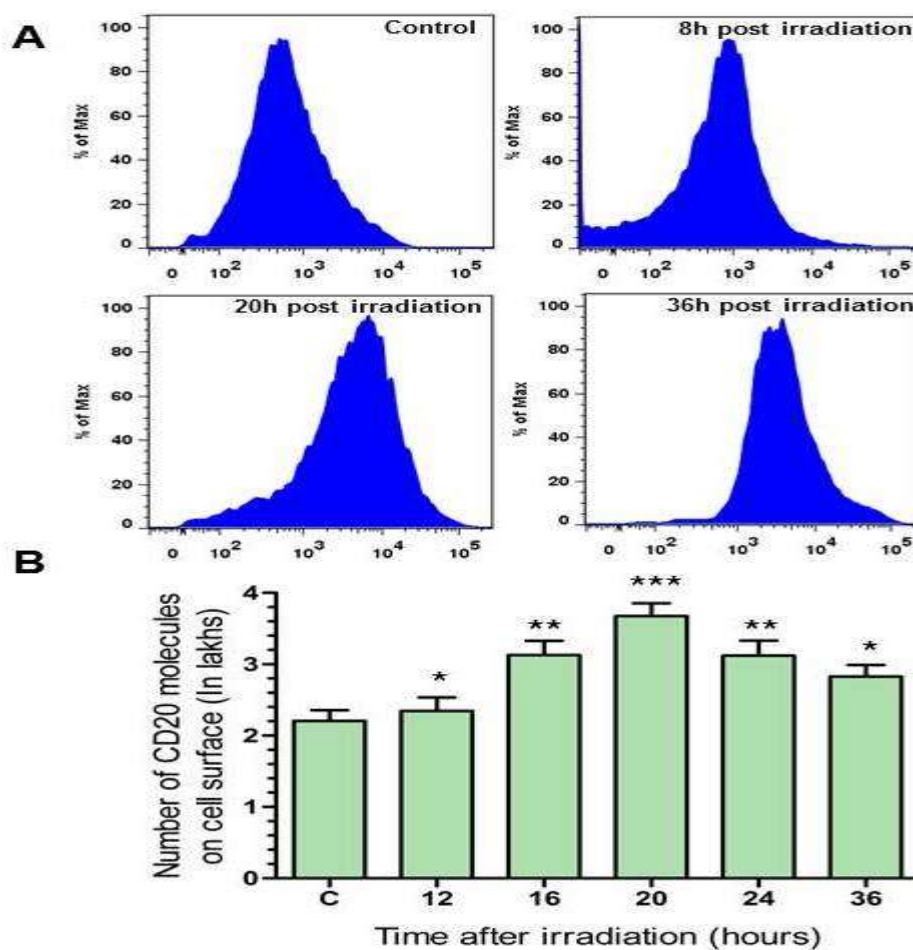


Fig. 1: Flowcytometrically measurement of CD20 expression on cell surface

(A) The index histograms are showing levels of CD20 at different time intervals with respect to 0hr followed by 0.5Gy radiation exposure. The levels of CD20 at cell surface levels were calculated using quantiBRITE beads and expressed as numbers of CD20 molecules/ cell. (B) The surface expression of CD20 molecules was calculated using standard calibration curve and expressed as numbers of CD20 molecules/ cell. Significance was calculated using Student t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ control vs radiation groups).

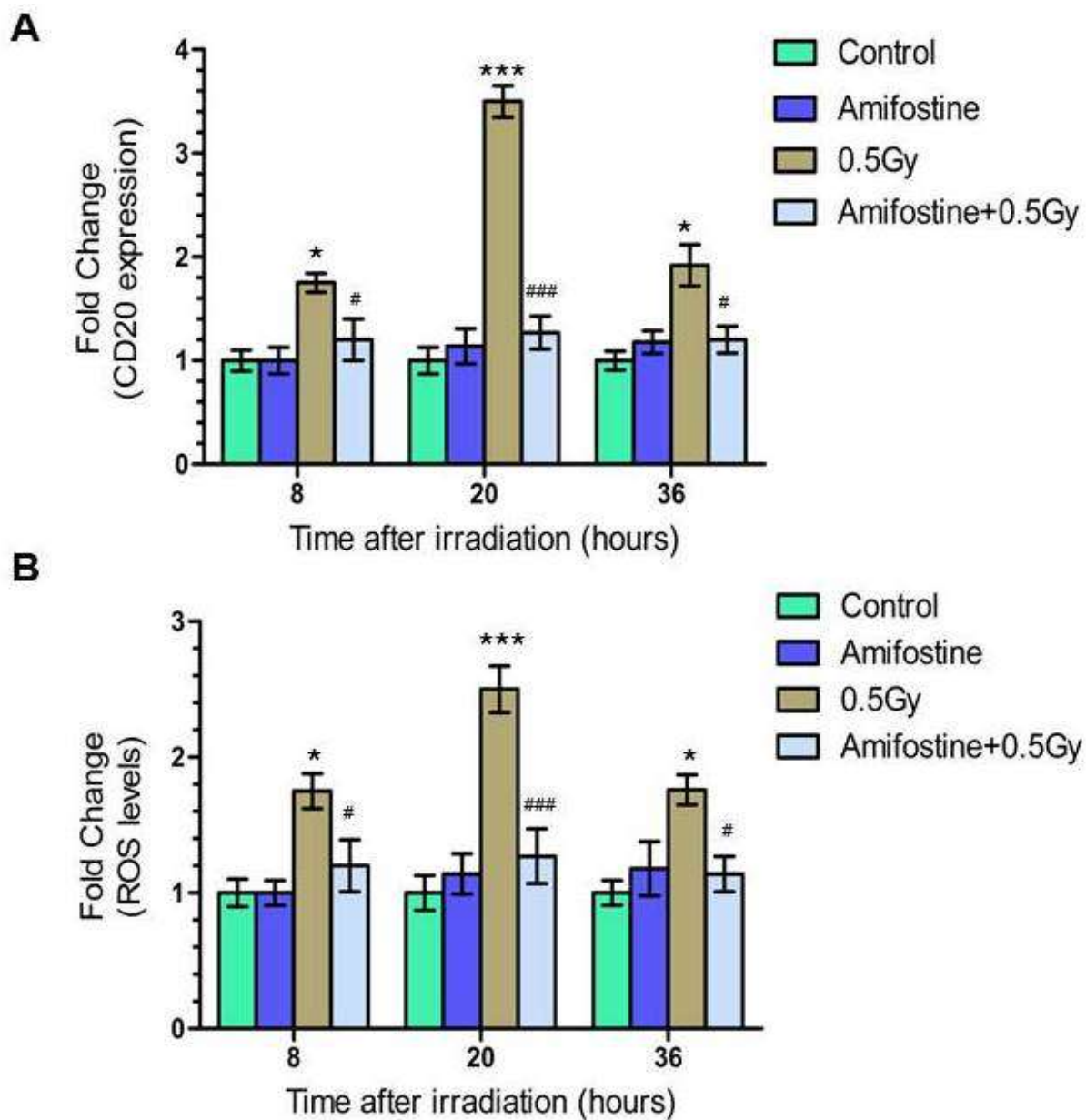


Fig. 2: Redox regulation of CD20 expression

(A) The kinetics of CD20 expression and (B) changes in ROS generation were measured with respect to untreated control by flow cytometry after different treatments with amifostine, radiation, or a combination of amifostine with radiation (Daudi; 0.5Gy). Cells were treated with amifostine (0.1 µg/ml; 1 h) followed by irradiation and thereafter cells were washed to remove the amifostine as described under materials and methods. Expression of CD20 and ROS was measured by flow cytometry using PE conjugated anti-CD20 antibody and CM-H₂DCFDA, respectively.

The data were analysed using Flow Jo 7.5.6 and the results are expressed as the mean fluorescence for ROS generation or CD20 expression. The statistical comparisons were done using ANOVA (**p*0.05, ***p*0.01, ****p*0.001 for control vs radiation, #*p*0.05, ###*p*0.001 for radiation vs amifostine+radiation).

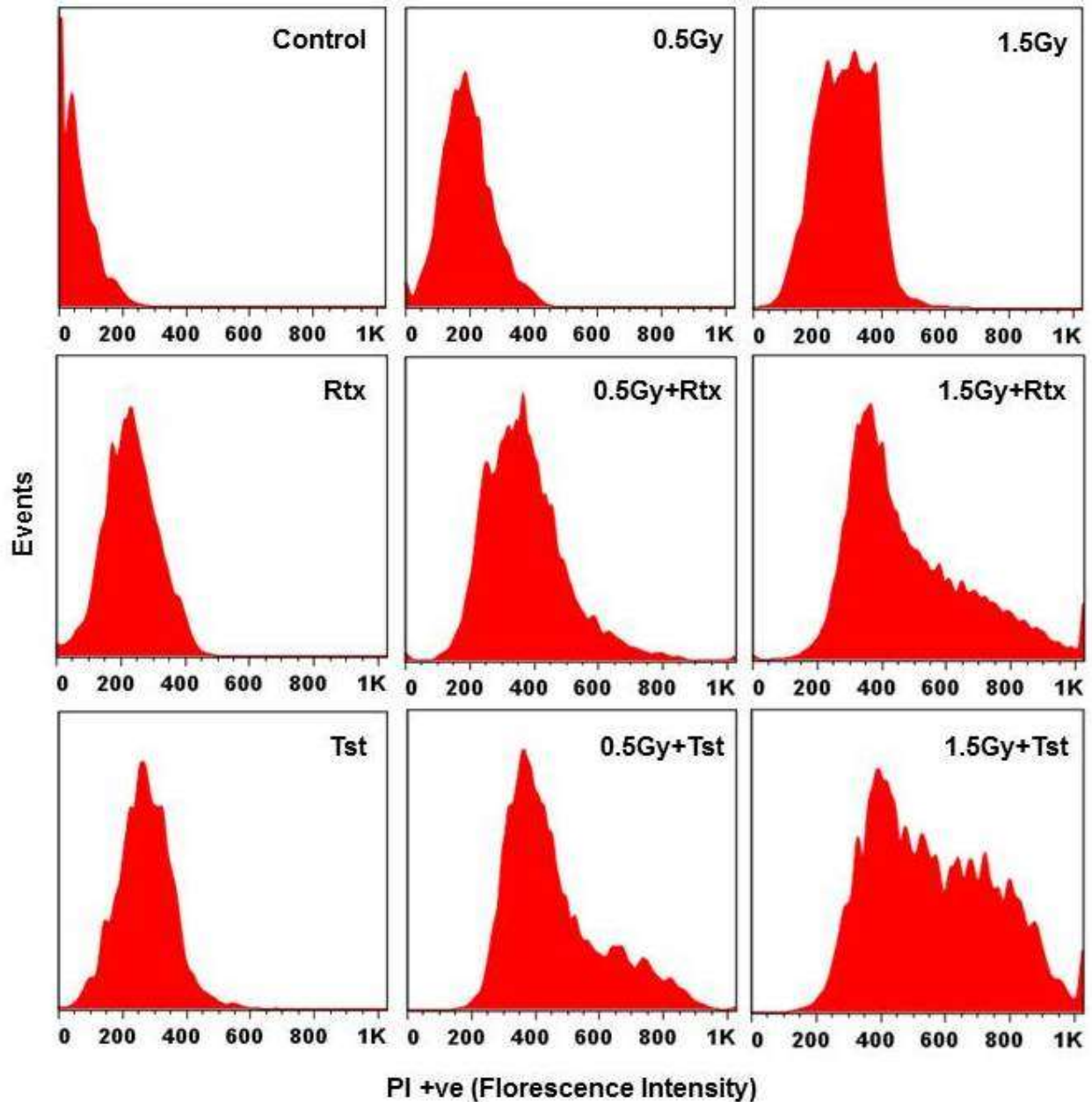


Fig. 3: Anti-CD20 mAbs induced an apparent cell death

(A) The overall cell death was measured using PI uptake by flowcytometrically. PI is membrane impermeable, generally excluded from viable cells and therefore, commonly used for identifying alterations in biological membrane and thereby death of cells in a population. Index histograms are showing cell death (PI +ve) populations.

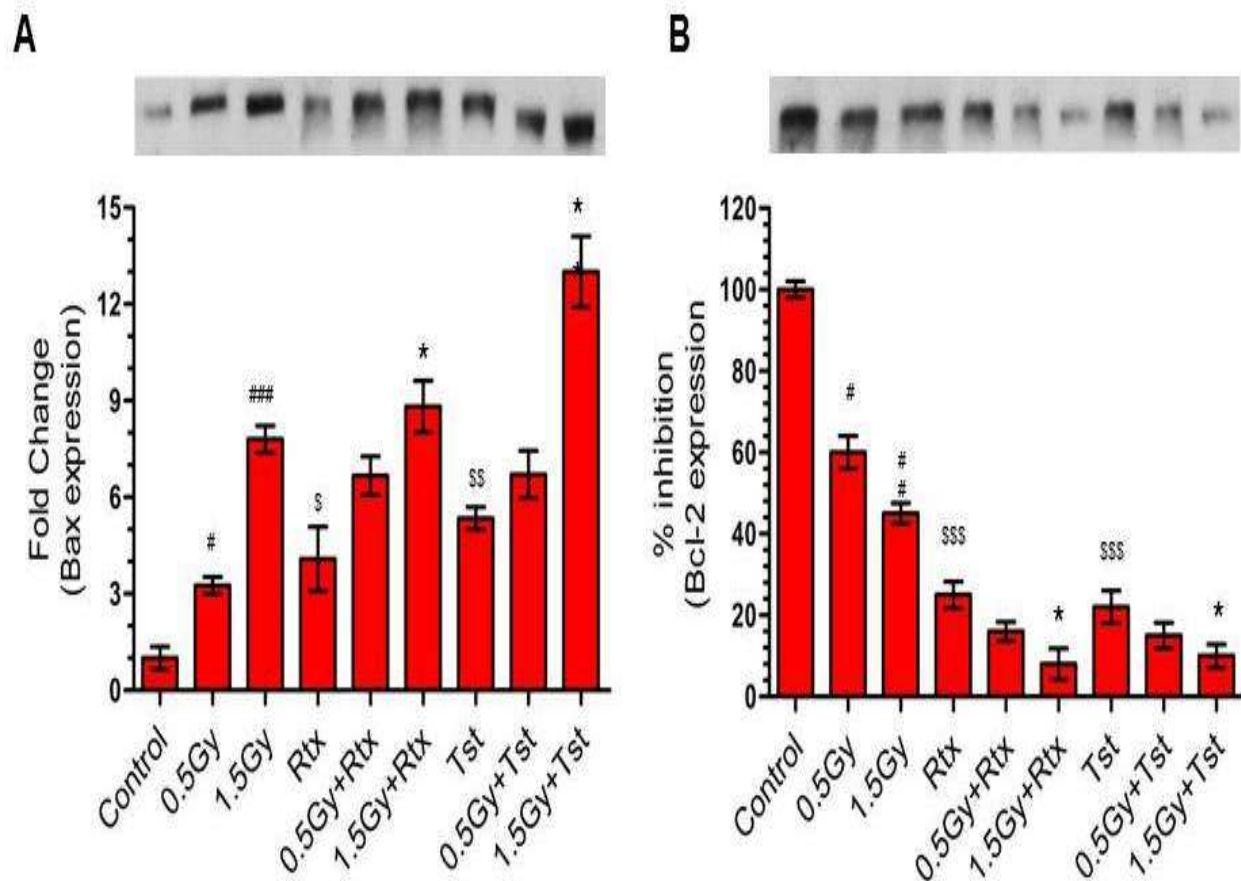


Fig. 4: The ratio of changes in Bcl-2/ Bax expression

For measurement of anti-apoptotic and pro-apoptotic proteins expression cells were lysed and equal quantity of proteins loaded and resolved in a 12% Tris-HCl gel, followed by transfer onto nitrocellulose membrane, and immunoblotted for both Bcl-2 and Bax (A) Bcl-2 protein protects the cell against cell death. The fold inhibition in Bcl-2 expression was measured from densitometric mean \pm SD and statistical analysis was performed using ONE way ANOVA. Significant values represented as; # p <0.05 for control vs 0.5Gy, ### p <0.01 for control vs 1.5Gy, \$\$\$ p <0.001 for control vs Rtx or Tst, * p <0.05 for Rtx vs 1.5Gy+Rtx or Tst vs 1.5Gy+Tst. (B) Bax is a pro-apoptotic protein. The fold increase in Bax expression was measured from densitometric mean \pm SD and statistical analysis was performed using ONE way ANOVA. Significant values represented as; # p <0.05 for control vs 0.5Gy, ### p <0.001 for control vs 1.5Gy, \$ p <0.05 for control vs Rtx, §§ p <0.01 for control vs Tst, * p <0.05 for Rtx vs 1.5Gy+Rtx and ** p <0.01 for Tst vs 1.5Gy+Tst.

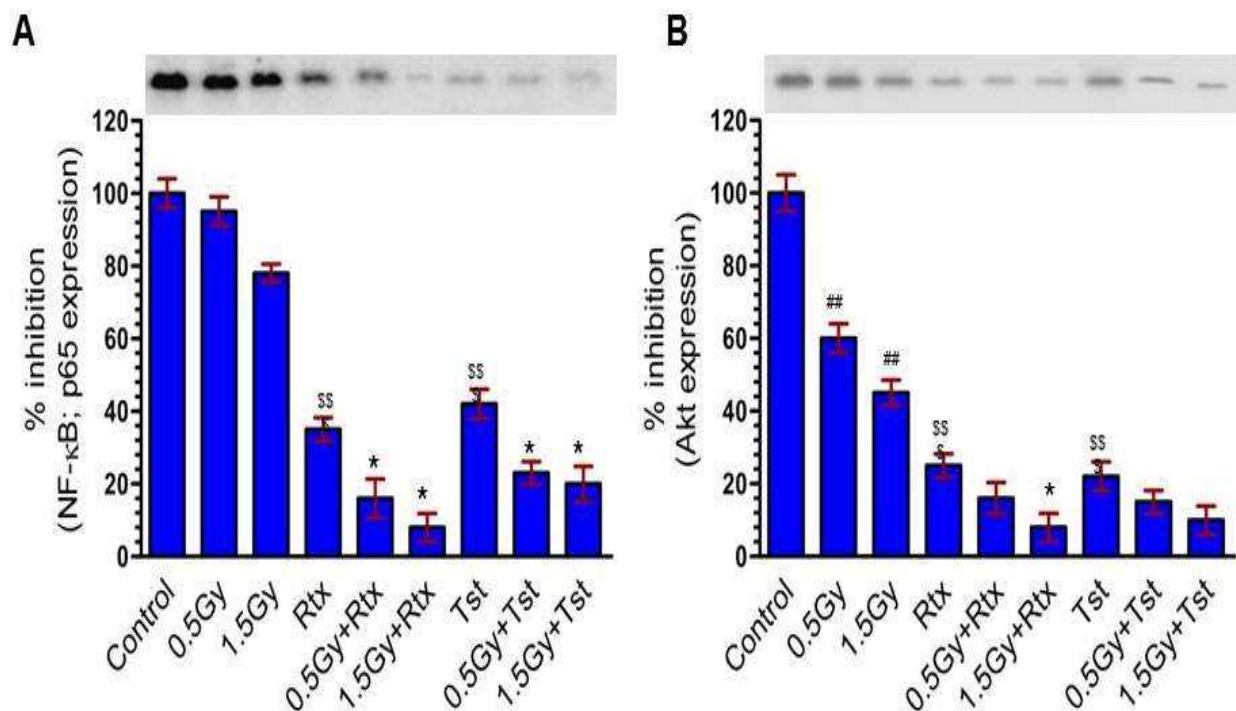


Fig. 5: The anti-CD20 mAbs induced inhibition of pro-survival and anti-apoptotic signaling
 For measurement of inhibition of pro-survival (NF-κB) and anti-apoptotic (Akt) signalling, p65 and Akt expression were measured respectively. (A) NF-κB is a widely accepted pro-survival signalling pathway. The percent inhibition in NF-κB (p65) expression was measured from densitometric mean ± SD and statistical analysis was performed using ONE way ANOVA. Significant values are represented as; \$\$\$\$ $p < 0.001$ for control vs Rtx or Tst, * $p < 0.05$ for Rtx vs 0.5Gy+Rtx or 1.5Gy+Rtx, * $p < 0.05$ for Tst vs 0.5Gy+Tst or 1.5Gy+Tst. (B) Akt is a prominent anti-apoptotic signalling pathway. The percent inhibition in Akt expression was measured from densitometric mean ± SD and statistical analysis was performed using ONE way ANOVA. Significant values are represented as; *** $p < 0.01$ for sham irradiated control vs 0.5Gy or 1.5Gy, \$\$\$\$ $p < 0.001$ for control vs Rtx or Tst, * $p < 0.05$ for Rtx vs 1.5Gy+Rtx

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