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Different radiosensitivity of CD4⁺CD25⁺ regulatory T cells and effector T cells to low dose gamma irradiation in vitro

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Abstract

Purpose—To determine the radiosensitivity difference of human Cluster of Differentiation (CD)4⁺CD25⁺ regulatory T cells (Treg) and effector T cells to low dose gamma ray and elucidate the underlying mechanisms in vitro.

Materials and methods—Blood samples were collected from five health subjects and five patients with advanced hepatocellular carcinoma (HCC). Treg and CD4⁺CD25⁺ T cells were selected using magnetic microbeads. The proliferative profiles, cytokine secretion, and differential expressions of apoptosis-related proteins in Treg and CD4⁺CD25⁺ T cells were compared using [³H]-thymidine incorporation, Luminex assay and flow cytometry when treated with various low doses of γ -ray.

Results—A dose-dependent reduction of proliferation in response to irradiation which paralleled the induction of apoptosis existed in Treg and CD4⁺CD25⁺ T cells. Treg were more radiosensitive to low-dose irradiation (0.94 Gray [Gy]) than effector T cells. The interferon- γ (IFN- γ) was significantly upregulated and interleukin 10 (IL-10) was significantly downregulated in irradiated Treg. An enhanced immune response to low dose gamma ray existed in the peripheral blood in patients with advanced HCC. Higher levels of active caspase-3, CD95, B cell lymphoma 2 (Bcl-2)-associated X protein (Bax) expression were observed in Treg compared to CD4⁺CD25⁺ T cells. In addition, gamma irradiation activated CD4⁺CD25⁺ T cells to express CD25.

Conclusions—These studies revealed that Treg were more radiosensitive than CD4⁺CD25⁺ T cells to low dose irradiation. Higher expressions of apoptosis-related proteins such as caspase-3, CD95 and Bax were observed in Treg when compared to CD4⁺CD25⁺ T cells. Our results suggest that treatment with low doses of gamma irradiation may be a viable strategy to enhance immune response in patients with advanced HCC.

Keywords

gamma irradiation; regulatory T cells; hepatocellular carcinoma; apoptosis; radiosensitivity

Introduction

Radiation therapy (RT) is the medical use of ionising radiation as part of cancer treatment to kill malignant cells and shrink tumours. It has been used alone or in combination with other therapies such as surgery, chemotherapy, immunotherapy, for both curative and palliative cancer treatment. Traditionally, RT has been viewed as having immunosuppressive effects due to the radiosensitivity of effector lymphocytes, but this concept has been challenged (Cole 1986, James et al. 1989, Wasserman et al. 1989). RT can theoretically enhance anti-tumour immunity via increasing the expression of tumour-associated antigens, inducing immune-mediated targeting of the tumour stroma, and diminishing regulatory T cell activity. Recent evidence suggests that RT may also activate effectors of innate immunity through Toll-like receptor (TLR)-dependent mechanisms, thereby augmenting the adaptive immune response to cancer (Roses et al. 2008). Thus RT can be better-characterised as having immunomodulatory properties that can allow its use as adjunct to immunotherapy (McBride et al. 2004).

The efficacy of RT in the treatment of various tumours is well established. While radiation induced tumour regression is largely the result of direct damage to radiosensitive tumour cells, evidence also points to a number of additional immune-mediated mechanisms. For example, suppressor T cells may be more radiosensitive than their effector counterparts with tumour-specific effector T cells being relatively radio-resistant (North 1984, 1986, Dunn and North 1991). Most recent evidence showed that low dose total body irradiation selectively reduced the population of Cluster of Differentiation (CD)4⁺ CD25⁺ forkhead box P3 (Foxp3)⁺ regulatory T cells and enhanced antitumour immunity in murine model (Liu et al. 2010).

Ionising radiation induces diverse effects on cell survival, apoptosis, proliferation and differentiation depending on the dosage and target cell (Jonathan et al. 1999). High dose of gamma irradiation often results in massive DNA damage that involves double-strand breaks and subsequent cell death. Low dose of gamma irradiation induces reactive oxygen species (ROS) and the activation of specific intracellular signaling pathways and transcription factors leading to proliferation and differentiation of target cells (Kasid et al. 1996, Lander et al. 1997, Finkel 1998). Therefore, gamma irradiation can modulate immune response via its variable effects on immune cell survival and differentiation (Shankar et al. 1999, Rho et al. 2004, Liu 2007, Shan et al. 2007, Scott 2008).

CD25⁺CD4⁺ regulatory T cells (Treg) comprise 5–10% of the circulating CD4⁺ T cell population and suppress immune responses. A large body of experimental data suggest an essential role played by these cells in self-tolerance, transplantation, allergy and tumour/microbial immunity (Wing et al. 2006). Currently, many investigators are pursuing strategies to modulate the function or the number of Treg, which may offer a means to regulate host immunity for therapeutic effect in autoimmune diseases and cancers (Zou 2006). In murine tumour models, it has been shown that depletion of CD4⁺CD25⁺ cells by anti-CD25 monoclonal antibody (mAb) administration (Jones et al. 2002, Li et al. 2003), or adoptive cell transfer after CD4⁺CD25⁺ cell depletion (Shimizu et al. 1999, TanaKa et al. 2002), results in significant antitumour activity.

Ionising gamma radiation is commonly used as a method of decreasing lymphocyte numbers via cytotoxicity for the purpose of bone marrow transplantation (BMT). However, previous investigators have also suggested that low-dose whole-body irradiation results in immune enhancement in the treatment of chronic lymphocytic leukemia (CLL) and lymphoma (Safwat 2000a, 2000b). Accumulating evidence shows that gamma irradiation influences the phenotype and function of immune cells (Cao et al. 2004, 2009, Reuben et al. 2004, Merrick

et al. 2005). Our previous work suggested that gamma irradiation inhibited proliferation and suppressive ability of Treg with dose-dependent decrease of FOXP3 expression (Cao et al. 2009). Based on our and the work of other groups (Liu et al. 2010), we hypothesise that differential radiosensitivity and apoptosis-related proteins expression exist at varying doses of gamma radiation between human Treg and effector T cells. To explore these questions, we initially compared the radio-sensitivities of human Treg with CD4⁺CD25⁻ T cells to different doses of gamma radiation. We then characterised the differential expression of apoptosis-related proteins in Treg and CD4⁺CD25⁻ T cells after gamma radiation. Finally, we evaluated the impact of low doses of gamma radiation on effector T cell responses in whole peripheral blood from patients with advanced hepatocellular carcinoma (HCC).

Materials and methods

Human subjects

Whole peripheral blood samples were collected from patients with HCC as previously described (Cao et al. 2007), using a protocol approved by the University of Florida Institutional Review Board (IRB). Patients were predominantly male, age ranging between 50 and 65, and had advanced/inoperable HCC (multifocal disease or vascular invasion).

Antibodies

The following monoclonal antibodies were used: CD4-Allophycocyanin (APC), CD4-Fluorescein (FITC), CD25-Phycoerythrin (PE), CD25-Chromomycin (CyC), CD95-FITC, Caspase-3-FITC (all from BD Biosciences, San Jose, CA, USA), B cell lymphoma 2(Bcl-2)-PE, Bcl-2-associated X protein (Bax)-PE, B-cell lymphoma-extra large (BclxL)-PE (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Isotype-matched antibodies were used with all samples.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte-enriched buffy coats obtained from healthy blood donors as previously described (Cao et al. 2007). Blood was diluted 1/1 with phosphate buffered saline (PBS) (without calcium or magnesium), layered on Ficoll-Paque and centrifuged for 30 min at 400 g. The mononuclear cell-rich layer was removed, washed twice with PBS, and resuspended in complete Roswell Park Memorial Institute (RPMI) 1640 medium.

CD4⁺ T cells were purified from PBMC using the human EasySep™ CD4⁺ T Cell Enrichment Cocktail (Stemcell Technologies Inc., Vancouver, BC, Canada). The negatively selected CD4⁺T cells were incubated with anti-CD25 microbeads (30 µg per 10⁸ cells) and subjected to magnetic separation with the magnetic selection (MS) column (all from Miltenyi Biotec Inc., Auburn, CA, USA) to obtain the purified CD4⁺CD25⁺ T cells from the column and the eluted negative CD4⁺CD25⁻ T cell fraction as previously described. The purity for CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells was more than 90% and 95%, respectively, by flow cytometry. Monocytes were positively selected from PBMC by using anti-CD14 microbeads (Miltenyi Biotec) following the manufacture's instruction and irradiated (30 Gy) for further use. The purity for CD14⁺ monocytes was more than 90%.

Gamma irradiation of cells

Purified CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were divided into several equal parts. One part was not irradiated; the others were γ -irradiated at different dosages with a ¹³⁷Cs source (Gammacell 200, Energy Atomic of Canada Ltd, Ontario, Canada) at a dose rate 4.8 Gy/min. The medium was immediately removed and the cells were resuspended in complete

RPMI 1640 medium. Cell viability of 100% was determined by trypan blue exclusion staining for all experiments.

Measurement of apoptosis and apoptosis-related proteins

After gamma irradiation, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were cultured in fresh complete RPMI 1640 medium overnight, washed two times with PBS, blocked with 2% human AB serum in PBS for 7 min at room temperature, and then incubated with CD95 for extracellular staining, or with caspase-3, Bax-PE, Bcl-2-PE, Bcl-xL-PE for intracellular staining after fixing and permeabilising in Cytotfix/Cytoperm buffer (BD biosciences).

Apoptosis was measured using the Annexin V-FITC Apoptosis detection Kit (BD Pharmingen, San Diego, CA, USA). Briefly, after overnight culture, washed cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. Acquisition and data analysis were performed with FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Inc., San Jose, CA, USA). The results were expressed as percentages of cells that stained positively.

Proliferation assays

CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (1×10^5 /well) were respectively co-cultured with monocytes (irradiated, 30 Gy) at ratio of 1:1, or 100 μ l of four times culture medium diluted HCC patients whole blood samples were seeded in 96-well U-bottom plate after sham irradiation or irradiation by different doses of γ -ray. For Treg 'add-back' experiments, the irradiated PBMC (1×10^5 /well) were co-cultured with or without the same dosage γ -ray-irradiated purified autologous CD4⁺CD25⁺ T cells (5×10^4 /well) from the same batch of PBMC. As a control group, the irradiated CD4⁺CD25⁺ T cells by different doses of γ -ray were cocultured with irradiated PBMC by 30 Gy of γ -ray. All experiments were done in triplicate and cultured for two days in the presence of phytohemagglutinin (PHA) (5 μ g/ml), supernatants were collected and stored at -86°C for cytokines assay, and then 1.0 μCi ^3H -thymidine was added to cultures for another 18 h. The radioactivity of cells was measured and expressed as the mean counts per min (cpm).

Cytokines measurement by Luminex

The levels of interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), interferon (IFN) and tumour-necrosis factor (TNF) in culture supernatants were determined using Luminex and MILLIPLEX[®] MAP human cytokine/chemokine kit obtained from Millipore Corporation (Billerica, MA, USA) and the procedures were conducted as recommended by the supplier. Briefly, a standard curve for each of the target proteins was prepared by serial dilution in assay buffer. Standards and samples were incubated in a 96-well filter-bottom plate with target-specific microparticles for 16–18 h at 4°C overnight incubation with shaking. Following the incubation, microparticles were washed twice using the wash buffer provided in the kit and a vacuum manifold. Biotinylated antibodies were added and incubated for 1 h, and detected using a streptavidin-phycoerythrin conjugate. Following extensive washing, samples were read on a Luminex 100 plate reader. The MFI data of all the samples were analysed using 5-parameter logistic curve-fitting method and the concentrations calculated by comparison to the appropriate standard curve were expressed as median and standard derivative (SD).

Statistical analysis

Paired comparisons (irradiated versus non-irradiated) were done by Wilcoxon's signed rank test; the other data were compared using the non-parametric Wilcoxon's test. The

probability (*P*)-values quoted are two-tailed and differences are considered statistically significant at *P* = 0.05. Throughout the text, data are expressed as mean ± SD.

Results

CD4⁺CD25⁺ Treg cells are more radiosensitive than CD4⁺CD25⁻ T cells to low dose gamma irradiation

To compare the relative sensitivity of Treg with that of effector T cells after gamma radiation, the first important thing is to get the real human Treg cells distinguishing from activated effector cells. Up to now, due to the lack of specific markers for identification and isolation of human Treg cells, it is very difficult to get genuine human Treg cells without contamination of activated effector cells. The functional analysis that showed they have suppressive ability is the only reliable way to identify human Treg cells. As shown in Figure 1A and B, our selected CD4⁺CD25⁺ T cells can be viewed regulatory T cells with higher FOXP3 expression and suppressive capability. Compared to its counterparts CD4⁺CD25⁻ T cells, the isolated CD4⁺CD25⁺ T cells have much lower proliferation capability in response to the stimulation of PHA (Figure 1B, 1C) and are more radiosensitive than CD4⁺CD25⁻ T cells to low dose of γ -ray. After three days coculture with irradiated autologous monocytes, as shown in Figure 1C, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell proliferation was inhibited by γ -ray in a dose-dependent manner. However, these two cell populations have different responses to low dose gamma irradiation. At doses of 0.94 Gy, the proliferation of irradiated CD4⁺CD25⁺ T cells (Treg 0.94) are significantly inhibited (Treg 0.94 vs. Treg 0: 11769 ± 788 cpm vs. 22414 ± 1306 cpm, *P* = 0.0023), in contrast to CD4⁺CD25⁻ T cells (T 0.94 vs. T 0: 47706 ± 8163 cpm vs. 57336 ± 7617 cpm, *P* = 0.1729). At doses above 1.875 Gy, proliferation is markedly inhibited in both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, respectively. At a higher dose of 15 Gy, the levels of cell proliferation inhibition for these two cell populations are similar. These results indicate that, compared to effector CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ Treg cells are more radiosensitive to low dose of γ -ray (0.94 Gy).

Enhanced IFN γ and reduced IL-10 secretion in response to low dose gamma ray were observed in Treg

In order to determine whether gamma irradiation has any effect on the cytokine profiles of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, we assessed the ability of Treg and T cells to produce IL-1, IL-2, IL-4, IL-6, IFN γ and TNF α in response to low doses of gamma ray. In contrast to the control groups (0 Gy), as shown in Table I, the significantly increased secretion of IFN γ in response to 0.94 Gy and 1.87 Gy of γ -ray were observed in both cell populations. Meanwhile, the secretion of IL-1, IL-6, IL10 and TNF α were significantly down-regulated in Treg, but not in T cells.

Higher baselines for apoptosis and apoptosis-related proteins expression exist in Treg

Based on the above results, we next examined whether there was differential level of apoptosis in non-irradiated Treg and CD4⁺CD25⁻ T cells at baseline. As shown in Figure 2A, the percentage of early apoptotic cells (Annexin V⁺/PI⁻) is much higher in Treg than in T cells (29.02% in Treg vs. 11.74% in T cells). A dose-dependent increase in apoptosis is observed in both cell populations, which parallel their proliferation profiles (Figure 1B). In light of these results, we postulated that a differential expression of apoptosis-related proteins existed between Treg and effector T cells. To test this hypothesis, we then examined the expressions of CD95, caspase-3, Bax, Bcl-2 and Bcl-xL in Treg and effector T cells. As shown in Figure 2C, D, E and F, the expression of CD95, caspase-3 in non-irradiated Treg are, respectively, 1 and 1.9 times higher than in non-irradiated T cells. The effects of gamma irradiation on CD95, caspase-3 expressions in Treg and T cells were

different. After irradiation, the active caspase-3 expression in two cell populations increased in a dose-dependent manner, but the CD95 expressions remained stable. The proapoptotic Bax protein expression was enhanced corresponding to apoptosis induction (Figure 2G, 2H), but no changes in expression were observed for the anti-apoptotic protein Bcl-2 and Bcl-xL (data not shown).

Previous studies have shown that total body irradiation (TBI) of mice can induce an increase in activated T cells (Miller et al. 2003). To verify the irradiation-induced activation in human T cells, we monitored the activation state of T cells after irradiation. The results in Figure 2I show that gamma irradiation induced a significant increase in the number of CD4⁺ T cells expressing CD25 marker (activated T cells) relative to non-irradiated CD4⁺ CD25⁻ T cells.

Enhanced immune response to mitogenic stimulation exists in HCC whole peripheral blood after low dose of gamma irradiation

Based on the results that CD4⁺CD25⁺ T cells are more radiosensitive than effector CD4⁺CD25⁻ T cells to γ -ray, and that low dose gamma ray can stimulate IFN γ secretion and activate CD4⁺CD25⁻ T cells, we wanted to see if immune responses can be enhanced using ex vivo irradiation-induced inhibition of Treg with low dose radiation. We chose to evaluate low doses of gamma irradiation using whole peripheral blood from patients with advanced HCC that are well described to have high levels of peripheral Treg implicated in cancer development and progression (Cao et al. 2007). As shown in Figure 3A and 3B, stronger immune responses are observed at doses of 0.94 Gy gamma irradiation ($p < 0.05$).

To mimic the situation of Treg in tumour environment, and to further confirm that the enhanced immune response resulted from the inhibition of Treg proliferation to low dose irradiation, we added purified irradiated CD4⁺CD25⁺ T cells (5×10^4 /well) back to the same dose-irradiated autologous PBMC (1×10^5 /well) from control patients. The data in Figure 3C and 3D showed an enhanced response to PHA stimulation in Treg-added PBMC after irradiation at dose of 0.94 Gy γ -ray. Together, these results suggest low dose irradiation (0.94 Gy) enhances the cellular immune response to mitogenic stimulation via the inhibition of Treg proliferation.

Discussion

An improvement in our understanding of the stimulatory effect of low dose radiation (LDR) may reveal novel mechanisms and offer new treatment options. Currently, low-dose total-body irradiation (LTBI) is a valid option in the treatment of chronic lymphocytic leukemia (CLL) and lymphoma (Dobbs et al. 1981, Richaud et al. 1998). A typical treatment schedule consists of 0.1- to 0.25-Gy fractions given several times a week until a cumulative dose of approximately 1.5–2 Gy is reached. Despite no recommendation to metastatic melanoma patients due to its poor clinical efficacy (Safwat et al. 2005), LTBI is successful in inducing long-term remissions and has been shown to be as effective as the chemotherapy to which it has been compared in lymphoma (Safwat 2000a). However, the mechanism of this effect is unknown.

The efficacy of LTBI could be partly attributed to a radiation-induced immune enhancement rather than to direct killing of tumour cells by radiation. It has been demonstrated that radiation-induced changes in immune activity follows an inverse J-shaped curve, i.e., low dose stimulation and high dose suppression (Liu 2007). This type of immune enhancement could be induced by two mechanisms: a differential elimination of the suppressive T subset of lymphocytes or an augmentation of the immune response through direct and/or indirect stimulation of T lymphocytes. One of the earliest reports about the immune-stimulatory

effects of low-dose irradiation was of an enhanced primary antibody response if 0.05–0.25 Gy is administered after immunisation. The authors hypothesised that this was due to the elimination of a radiosensitive subpopulation of suppressor T cells (Safwat, 2000a, 2000b). These early studies, however, were done without the use of modern techniques to characterise the fate of different subtypes of lymphocytes in LTBI, and also most recent experiments explored the radiosensitivity of murine CD4⁺CD25⁺ Treg to gamma ray are controversial (Liu et al. 2010, Qu et al. 2010).

The role of CD4⁺CD25⁺ Treg in suppressing anti-tumour immune activity is subject to considerable interest. Treg maintain immunological tolerance by suppression of autoreactive T cells. Accumulating evidence indicates that depletion of Treg results in significant anti-tumour activity (Jones et al. 2002, Li et al. 2003). Based on these findings, enhancement of anti-tumour immunity by the removal of suppressor cells such as Treg has become an attractive strategy for augmenting more traditional cancer treatment (surgery and chemotherapy). It was shown that low dose total body irradiation selectively decreased the proportion and absolute number of Treg and enhanced antitumour immunity in murine model (Liu et al. 2010). In the present study, we firstly report a systematic investigation into the radiosensitivities of human Treg to gamma irradiation, the differential apoptotic status and apoptosis-related proteins expression in Treg and CD4⁺CD25[−] T cells. Our data show that Treg displaying much higher apoptotic baseline and apoptosis-related proteins expression are more radiosensitive than its effector counterpart CD4⁺CD25[−] T cells (Figure 1).

The immune system responds to ionising radiation with distinct characteristics depending on multiple factors such as dose and dose rate, tissue and cell types. Overall, immune cells are susceptible to radiation-induced damage and readily undergo apoptosis in response to small doses of radiation. Cellular apoptosis is critically regulated by various intracellular and extracellular signaling mechanisms. CD95 (Apo-1/Fas) is a homotrimeric tumour necrosis factor receptor (TNFR) family member characterised by the presence of a death domain in its cytoplasmic tail. CD95-mediated apoptosis is an important process with enormous physiological and pathophysiological impact and cell-type-specific features. CD95 molecules have been implicated in the maintenance of self-tolerance and T-cell homeostasis by transmitting apoptotic signals to repeatedly activated antigen-specific T cells, as well as to antigen-presenting dendritic cells (DCs) and activated B cells. It has also been identified as an important mediator of T-cell depletion in lymphopenic individuals (Schütze et al. 2008). Our data showed that CD95 expression in non-irradiated Treg is much higher than that in non-irradiated T cells, but not affected by gamma irradiation (Figure 2).

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3 is a key executioner of apoptosis, which is activated by initiator caspase such as caspase-9. These activated caspases cleave many cellular substrates, ultimately leading to cell death (Earnshaw et al. 1999, Porter and Jänicke 1999). We found that active caspase-3 protein expression in Treg is higher than in T cells, and is up-regulated by gamma irradiation in a dose-dependent manner. Thus, low dose of γ -ray-induced cell death in Treg and T cells was accompanied by an increase in the caspase-3, which then stimulates the molecular cascade of apoptosis (Figure 2).

The Bcl-2 family of protein comprising both anti- and pro-apoptotic members plays pivotal roles in regulating cell apoptosis (Finucane et al. 1999, Gross et al. 1999, Harris and Thompson 2000, Jung et al. 2001). In this family, the balance of Bcl-2/Bax in a cell is regarded as one of the crucial factors determining whether or not the cell will undergo apoptosis. Interestingly, we found a dose-dependent enhancement in the expression of pro-apoptotic protein Bax (Figure 2) and no change in the expression of anti-apoptotic protein

Bcl-2 (data not shown). These findings suggest that the Bcl-2/Bax balance shifted towards apoptosis induction in Treg after gamma irradiation.

In this work, an important finding to help explain the immune ‘stimulating’ effect of low dose irradiation is the differential impact on Treg vs. CD4⁺CD25⁻ cell population. Here we provide in vitro data to suggest that CD4⁺CD25⁺ T cells are more radiosensitive than autologous CD4⁺CD25⁻ effector T cells to low doses of γ -ray (Figure 1). Thus, the net balance for the immune system may be a relative decrease in Treg-mediated suppression and a net increase in effector T cell activity. The similar effect was confirmed in in-vivo that LTBI is a very attractive adjuvant strategy to enhance overall cancer immunotherapy or vaccine responses (Liu et al. 2010). The anti-tumour effects of LTBI could be explained by immune enhancement, induction of apoptosis, and intrinsic hypersensitivity to low-dose irradiation (Safwat 2000a, Liu et al. 2010). Possible mechanisms of immune enhancement are elimination of suppressor cells such as Treg and myeloid suppressor cells and augmentation of the immune response including natural killer (NK) cells, B cells and T cells activation, costimulatory molecules upregulation, IFN γ and IL-2 production and release, and enhanced proliferative activity of lymphocytes to mitogenic stimuli (Belka et al. 1999, (Hashimoto et al. 1999, Safwat 2000a, 2000b, Liu et al. 2001, Kipnis et al. 2004, Jin et al. 2007, Shan et al. 2007, Lin et al. 2008). In this report, our data also show that IFN γ secretion in response to low doses of γ -ray significantly increased in both Treg and T cells, and that immune enhancement is elicited in advanced HCC patient’s whole blood after irradiation with low dose of γ -ray (0.94 Gy), likely due to inhibition on CD4⁺CD25⁺ Treg proliferation and fostering the proliferation and activation of CD4⁺CD25⁻ T cells (Figure 2).

In summary, Treg show higher baseline expression of apoptosis-related proteins and sensitivity to low dose γ -ray compared to CD4⁺CD25⁻ effector T cells, and low dose gamma irradiation can induce activation of CD4⁺CD25⁻ T cells. Manipulation of CD4⁺CD25⁺ Treg in terms of their frequency and functional activity via gamma irradiation can be an adjuvant therapeutic measure for enhancing tumour immunity against HCC. These results provide insight into the mechanism of low dose radiation and offer new strategies for radiotherapy of cancer patients.

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Figure 1.

The differential effects of gamma irradiation on CD4⁺CD25⁺ and CD4⁺CD25⁻ cells. After sham irradiation or irradiation by different doses of gamma ray, CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were co-cultured with irradiated autologous monocytes (1:1) for three days in the presence of PHA (5 µg/ml), respectively. Cells proliferation were determined by [³H] thymidine incorporation. The results expressed the mean counts per min (cpm). (A) The purities and FOXP3 expression for the isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells; (B) The suppressive capability of CD4⁺CD25⁺ T cells; (C) One representative of three independent experiments to show the different proliferations of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in response to different doses of gamma ray; (D) Values represent the average ± SD of three separate experiments. **P* < 0.05

Figure 2.

The effects of gamma irradiation on apoptosis induction, apoptosis-related proteins expression in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, and on the activation of CD4⁺CD25⁻ T cells. After sham irradiation or irradiation by different doses of gamma ray, CD4⁺CD25⁺ or CD4⁺CD25⁻ cells cultured overnight and then incubated with anti-Annexin V, anti-CD95, anti-caspase-3, anti-Bax or anti-CD25 antibodies, respectively. Thirty thousand-gated events were collected on FACSCalibur with CellQuest software. Numbers in the figures represent the percentage of fluorescence-positive cells in corresponding areas. Values are representative examples of three separate experiments.

Figure 3.

The effect of low dose of gamma irradiation on enhancement immune response. As mentioned in *Materials and methods*, the enhanced immune response to the mitogen PHA (5 µg/ml) stimulation were observed in HCC patients peripheral blood (A and B) and in the in vitro culture system by using purified CD4⁺CD25⁺ cells to add-back to autologous PBMC (C and D) after sham irradiation or irradiation by low dose of gamma ray. In the Treg add-back experiments, after different doses of irradiation, the purified CD4⁺CD25⁺ cells and PBMC were divided equal two parts, the same dose -ray-irradiated CD4⁺CD25⁺ (5×10⁴/well) and PBMC cells (1×10⁵/well) mixed together and seeded in 96-well U-bottom plate for three days culture. The other part of cells used as control. (A) Data are from one of five HCC patients; (B) Values represent the average ± SD of five HCC patients (*n* = 5); (C) Data are from one of three independent experiments; (D) Values represent the average ± SD of three separate experiments (*n* = 3). Treg+PBMC: irradiated Treg by different doses of -ray cultured with the same dose-irradiated autologous PBMC; Treg+PBMC30 (control groups): irradiated Treg by different doses of -ray cultured with irradiated autologous PBMC (30 Gy); PBMC (control groups): irradiated autologous PBMC by different doses of -ray.

**p* < 0.05.

Table 1

Effect of gamma irradiation on cytokine secretion by CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ T cells.

	IL-1, pg/mL		IL-2, pg/mL		IL-4, pg/mL		IL-6, pg/mL		IL-10, pg/mL		IFN, pg/mL		TNF, pg/mL	
	T	Treg	T	Treg	T	Treg	T	Treg	T	Treg	T	Treg	T	Treg
OGY	39.0±1.8	49.7±2.0	18.2±0.7	4.2±0.4	4.9±0.4	2.7±0.7	758.6±34.1	989.7±45.3	23.9±1.2	25.6±1.9	215.3±13.1	86.7±3.6	145.9±16.7	50.3±2.9
0.94 Gy	35.4±1.1	41.1±1.7 ^a	15.8±0.7	3.9±0.6	5.1±0.8	4.8±0.8	704.9 ±33.8	842.3 ±42.3	18.4±0.9 ^a	18.8 ±1.0 ^a	256.6 ±14.2 ^a	115.6 ±4.3 ^a	121.2±12.9 ^a	49.2 ±2.3
1.87 Gy	43.3±1.5	34.1±1.0 ^{a,b}	21.9±0.9	3.6±0.5	7.1±0.9	3.6±0.7	869.6±41.5 ^b	535.7±29.8 ^{a,b}	23.7±1.0 ^b	15.8±1.1 ^{a,b}	460.4±15.0 ^{a,b}	134.9±4.6 ^{a,b}	188.2±15.3 ^{a,b}	35.3±1.1 ^{a,b}

Secretion of IL-1, IL-2, IL-4, IL-6, IL-10, IFN and TNF was determined in the supernatants of cultured CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ T cells after irradiation at different doses of gamma ray using commercially available human cytokine. Luminex assay kit as mentioned in *Materials and methods*. Values represent average ± SD of three independent experiments ($n = 3$).

^a vs. control group (0 Gy) $P < 0.05$;

^b 1.87 Gy group vs. 0.94 Gy group: $P < 0.05$.