

Original Article



Role of PLC-PIP2 and cAMP-PKA Signal Pathways in Radiation-induced Immune-suppressing Effect*

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Abstract

Objective The purpose of the present study was to observe the changes in CD4+CD25+Nrp1+Treg cells after irradiation with different doses and explore the possible molecular mechanisms involved.

Methods ICR mice and mouse lymphoma cell line (EL-4 cells) was used. The expressions of CD4, CD25, Nrp1, calcineurin and PKC- α were detected by flow cytometry. The expressions of TGF- β 1, IL-10, PKA and cAMP were estimated with ELISA.

Results At 12 h after irradiation, the expression of Nrp1 increased significantly in 4.0 Gy group, compared with sham-irradiation group ($P < 0.05$) in the spleen and thymus, respectively, when ICR mice received whole-body irradiation (WBI). Meanwhile the synthesis of Interleukin 10 (IL-10) and transforming growth factor- β 1 (TGF- β 1) increased significantly after high dose irradiation (HDR) ($> \text{ or } = 1.0 \text{ Gy}$). In addition, the expression of cAMP and PKA protein increased, while PKC- α , calcineurin decreased at 12h in thymus cells after 4.0 Gy X-irradiation. While TGF- β 1 was clearly inhibited when the PLC-PIP2 signal pathway was stimulated or the cAMP-PKA signal pathway was blocked after 4.0 Gy X-irradiation, this did not limit the up-regulation of CD4+CD25+Nrp1+Treg cells after ionizing radiation.

Conclusion These results indicated that HDR might induce CD4+CD25+Nrp1+Treg cells production and stimulate TGF- β 1 secretion by regulating signal molecules in mice.

Key words: Ionizing radiation; Neuropilin-1 (Nrp1); Regulatory T cells; Signal molecule

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INTRODUCTION

The immune-suppressing effect of high dose radiation (HDR, $> \text{ or } = 1.0 \text{ Gy}$) has been clearly demonstrated and confirmed both experimentally and in epidemiological

studies^[1-2]. Of note, regulatory T cells are central to the maintenance of immunological tolerance and suppressive control of excessive immune responses to foreign antigens^[3]. There is also accumulating evidence that HDR could induce the production of regulatory T cells^[4-6], and these studies mainly focus

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on the CD4+CD25+Treg cells or CD4+CD25+Foxp3+Treg cells after radiation. A recent study recognized neuropilin-1 (Nrp1) as one of the specific markers for Treg cells^[7]. However, the effects of ionizing radiation on CD4+CD25+Nrp1+Treg cells remain obscure up to now.

Nrp1 is a trans-membrane protein mainly involved in axonal guidance^[8], and angiogenesis as a receptor of class 3 semaphorins^[9] and VEGF (vascular endothelial growth factors)^[10]. It plays an important role in various biological processes, including angiogenesis, tumorigenesis, and immunological response. In the immune system, Nrp1 could be expressed on regulatory T cells (Tregs) as well as plasmacytoid dendritic cells (PDCs). It was also shown to be involved in the priming of T cells by DC^[11] and in regulation of the immunological synapse and response^[12-13]. In addition, Nrp1 is considered as a key player and has been added to the list of molecular mechanisms by which Treg cells exert their suppressive effects^[14]. Interestingly, Nrp1 could also play directly effects on tumor cells or indirectly mediate effects on tumor progression by affecting angiogenesis^[15-16]. Wiebke Hansen suggested that Nrp-1 acts as a key mediator of Foxp3+Treg cell infiltration into the tumor site resulting in a dampened anti-tumor immune response and enhanced tumor progression^[17]. Thus Nrp1 may act not only as a suppressing factor in antitumor immunity, but also as a promoting factor in tumor growth. NRP1 might represent an interesting link between tumorigenesis and immune tolerance and thereby a promising target for future therapies. In this study, we will focus on Nrp1+Treg, and explore the mechanisms of immune-suppressing effect after HDR.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs) and Reagents

The following monoclonal antibodies were used: CD4-PerCP-CyTM5.5, CD25-Phycoerythrin (PE), PKC α -Alexa Fluor[®] 488, (all from BD Biosciences, USA), Nrp1-Allophycocyanin (APC) purchased from R&D (USA), anti-calcineurin purchased from Abcam (USA), and goat-anti-rabbit IgG-FITC purchased from Tianjin Sanfrece Biotechnology Co., Ltd., China. Isotype-matched antibodies were used with all samples. Phorbol-12-myristate-13-acetate (PMA), 8-(Diethylamino) octyl-3, 4, 5-trimethoxybenzoate hydrochloride (TMB-8), Rp-Adenosine 3',5'-cyclic

monophosphorothioate triethylammonium salt hydrate (A165), and Forskolin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and Cells

Male and female ICR mice (body weight 20 \pm 2 g) were obtained from the Experimental Animal Center at Jilin University. The mice were divided into control and irradiated groups in randomized manner. All mice were maintained in a specific pathogen-free facility and were housed in micro-isolator cages containing sterilized feed, autoclaved bedding and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. Following sacrifice of the animals by decapitation, thymocyte and splenocyte suspensions were prepared as previously reported^[18]. 6 samples (3 male and 3 female ICR mice) were tested at each time/dose point.

EL-4 cells (mouse lymphoma cell line) were cultured in RPMI 1 640 medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 10 mmol/L Hepes (all from Hyclone, Logan, UT, USA). The cultured cells were divided into ten groups: control group, ionizing radiation (IR) group, PMA group, TMB-8 group, A165 group, Forskolin group, IR+PMA group, IR+TMB-8 group, IR+A165 group, and IR+Forskolin group. 3 samples were tested in each group.

Irradiation Protocol

For whole body irradiation (WBI), mice were placed in containers and subjected to irradiation using an X-ray machine (Dandong City Yasuyoshi Equipment Co, Ltd, China), at a dose rate of 11.87 mGy/min for doses of 0.05 to 0.2 Gy and 0.387 Gy/min for doses of 0.5 Gy and above. Sham-irradiated mice were used as controls. Radiation doses ranging from 0.0 to 6.0 Gy (0.0, 0.075, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 Gy) were given. At 12 h after WBI, the mice were sacrificed by decapitation, and then thymocytes and splenocytes were harvested. For *in vitro* irradiation, X-irradiation with the dosage of 4.0 Gy was given to EL-4 cells, and then the cells were cultured in glass Petri dishes at 37 °C in a CO₂ incubator for 48 h before assays.

Mechanistic Studies

Flow Cytometry The expression of CD4, CD25,

Nrp1, calcineurin, and PKC- α were determined by flow cytometry. Splenocytes, thymocytes, or EL-4 cells were incubated with an optimal concentration of fluorochrome-labeled mAbs for 45 min at 4 °C in the dark. Cells were washed twice with cold PBS (PBS with 0.1% BSA and 0.1% NaN₃) and resuspended in PBS with 0.1% BSA. Each sample was measured with FACS Calibur flow cytometer (Becton Dickinson) by counting at least 10 000 cells, and data were analyzed with FACS Diva software.

ELISA The production of TGF- β 1, IL-10, PKA, and cAMP were determined by ELISA after irradiation with different doses. The supernatants from EL-4 cells and the tissue homogenate from spleen and thymus were harvested. The TGF- β 1, IL-10, PKA, and cAMP were determined using ELISA kit (GBD, Co.), according to the manufacturer's instructions.

Statistical Analysis

All the statistical tests were performed using the SPSS for Windows 11.0 software package (SPSS, Chicago, IL, USA). Statistical analysis was performed

using the Student *t*-test (Figures 1, 2) and One-way ANOVA (Figures 3, 4). Differences between the means and the corresponding control value were determined, and *P*<0.05 was considered statistically significant.

RESULTS

Changes of the Nrp1 Receptor Expression on CD4+ Cells after Irradiation

To evaluate the effects of the Nrp1 receptor on CD4+ cells after irradiation, we evaluated the production of CD4+CD25+Nrp1+ cells in splenocytes and thymocytes. As seen in Figure 1, compared with sham-irradiation group, CD4+CD25+ cells increased significantly in the thymus and spleen following 12 h at 4.0 Gy X rays (A). Interestingly, an increase in the percentage of CD4+CD25+Nrp1+ cells was also found at 12 h after WBI of mice at a dose of 4.0 Gy X rays (B). These results showed that the over-expression of Nrp1 simultaneously with the increase of CD25 expression.

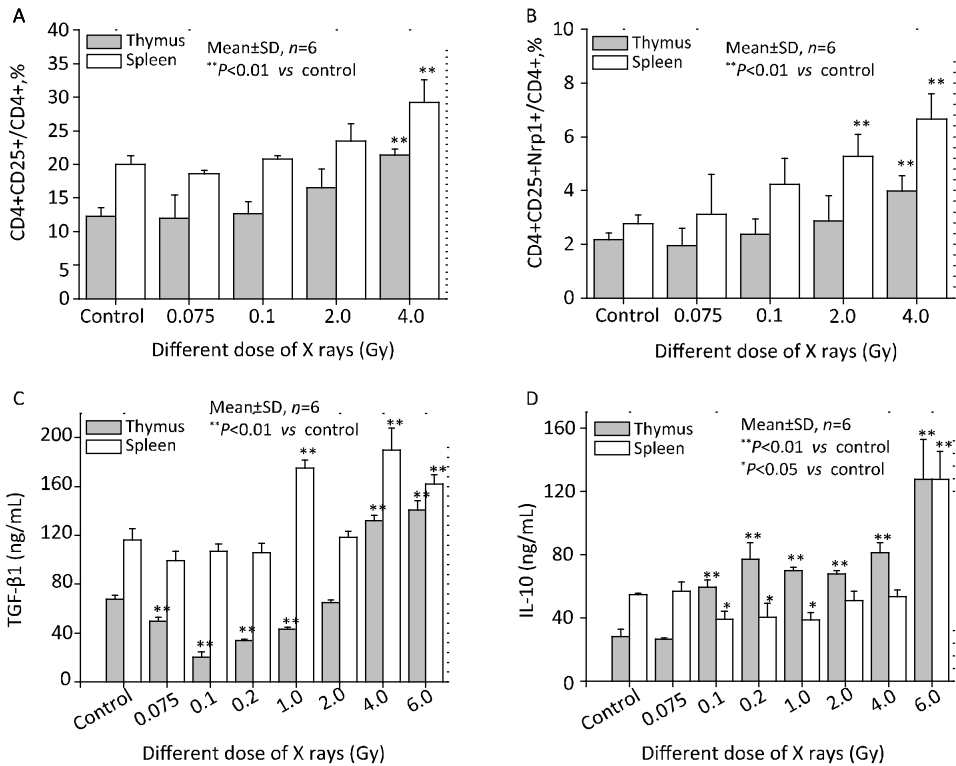


Figure 1. A, B: The percentage of CD4+CD25+ cell (A) and CD4+CD25+Nrp1+ cell (B) on thymocytes and splenocytes after WBI at different doses (0.075, 0.1, 2.0, 4.0 Gy) of X rays. C, D: The dose-effect relationship of TGF- β 1 (C) and IL-10 (D) synthesis and in thymus and spleen (D) a 12 h after 0-6.0 Gy (0.0, 0.075, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 6.0 Gy) X-rays.

Dose-course Changes of the IL-10 and TGF- β 1 Secretion

The suppressive mechanisms of Treg cells are under scrutiny, with multiple modes of action proposed, including mechanisms dependent on cell-cell contact and soluble factors. It has been suggested that immunosuppressive cytokines such as TGF- β 1 and IL-10 play an important role in the induction and/or maintenance of regulatory T-cells^[19]. In our study, we found that the secretion of TGF- β 1 increased markedly following 12 h at the exposure of 2.0 and 4.0 Gy in the thymus and spleen, while the TGF- β 1 decreased markedly with 0.075 to 0.5 Gy in thymus, and there was no change in the secretion of TGF- β 1 after radiation with 0.2 Gy in spleen (Figure 1C). Figure 1D showed similar changes in the IL-10 secretion.

Changes in Expression of Calcineurin, PKC- α , cAMP, and PKA after Irradiation with Different Doses

It is well known that T-cell activation requires two distinct signals (antigenic stimulation and co-stimulation), and these signals are delivered intracellularly by trans-membrane signal transduction via the TCR/CD3 complex coupled to PLC-PIP2 and

the cAMP-PKA system^[20]. We assessed whether the signal molecules could change after irradiation with different doses. After WBI of mice with different doses of X-rays, the expression of signal molecules related with PLC-PIP2 and the cAMP-PKA pathway were determined at 12 h. Differential results were observed as to the effect of low versus high doses of irradiation. As seen in figure 2, calcineurin (A) and PKC- α (B) related with PLC-PIP2 system as well as cAMP (C) and PKA (D) related with cAMP-PKA pathway showed different responses to low dose radiation versus HDR, with low dose stimulation and high dose suppression for calcineurin and PKC- α expression, while cAMP and PKA expression were stimulated with HDR and inhibited after low dose radiation.

Effect of the Changes in PLC-PIP2 or cAMP-PKA Signal Pathways on CD4+CD25+Nrp1+ Cells and TGF- β 1 after Irradiation.

The Figure 1 showed that the percentage of CD4+CD25+Nrp1+ cells and the TGF- β 1 were increased, while cAMP and PLA2 expression were stimulated with HDR, which were important in the cAMP-PKA signal pathways. While PKC- α and calcineurin were decreased after HDR (Figure 2). Thus,

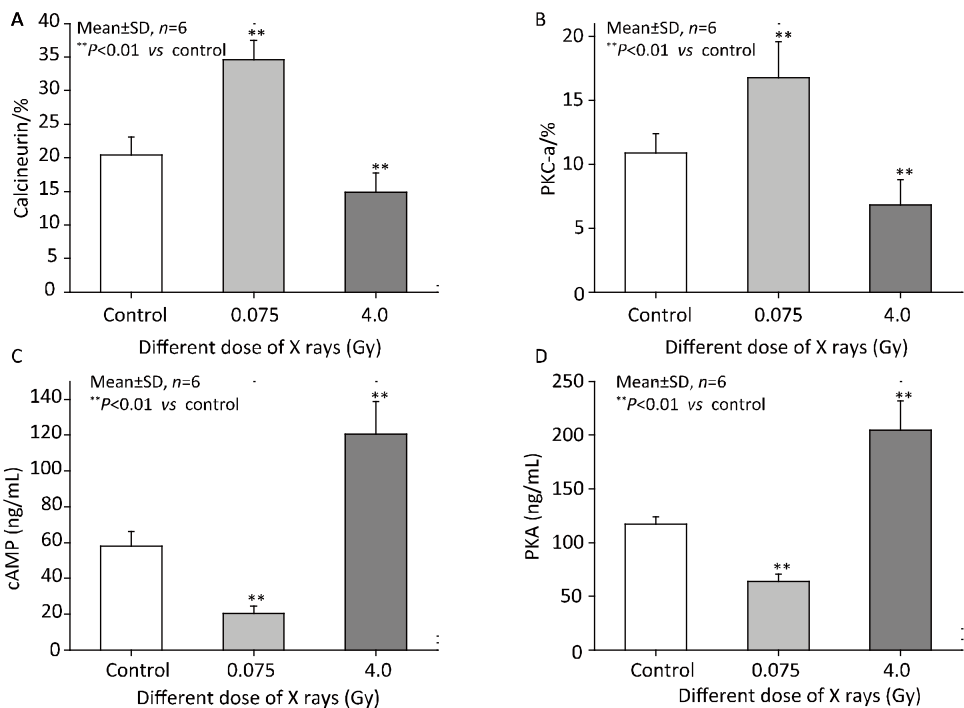


Figure 2. Expression of signal molecules related with PLC-PIP2 and the cAMP-PKA pathway in thymocytes followed WBI of mice with LDR (0.075 Gy) and HDR (2.0 Gy). A: calcineurin; B: PKC- α ; C: cAMP; D: PKA.

EL-4 (mouse lymphoma) cells were selected as samples in this study *in vitro*. In order to determine whether the number of CD4+CD25+Nrp1+ cells and the secretion of TGF- β 1 would be changed, PLC-PIP2 signal pathway was activated or blocked using pharmacologic activators (PMA) and inhibitors (TMB-8) respectively, and the cAMP-PKA signal pathway was activated or blocked with Forskolin and A165 respectively.

Changes in CD4+CD25+Nrp1+ Cells The ability of pharmacologic activators and inhibitors were tested in order to change the percentage of CD4+CD25+Nrp1+ cells by Flow Cytometry, and the percentage of CD4+CD25+Nrp1+ cells was increased markedly while activated by Forskolin and TMB-8, but strongly blocked by the A165 and PMA (Figure 3A). The effect of the pharmacologic on the

percentage of CD4+CD25+Nrp1+ cells after 4.0 Gy X rays irradiation was also examined next. Figure 3B showed that the percentage of CD4+CD25+Nrp1+ cells increased markedly after 4.0 Gy X rays irradiation, as compared with sham-irradiation. Interestingly, the Forskolin and TMB-8 could significantly increase the percentage of CD4+CD25+Nrp1+ cells in EL-4 cells after irradiation but this effect was not blocked with PMA or A165.

Changes in TGF- β 1 Secretion To determine whether the TGF- β 1 will be changed when the signal pathway was activated or blocked. We investigated the TGF- β 1 using ELISA. We found that the TGF- β 1 increased significantly in EL-4 cells with Forskolin and TMB-8, while PMA could inhibit the TGF- β 1, as compared with the control group (Figure 3C). We next studied the effect of the pharmacologic on the

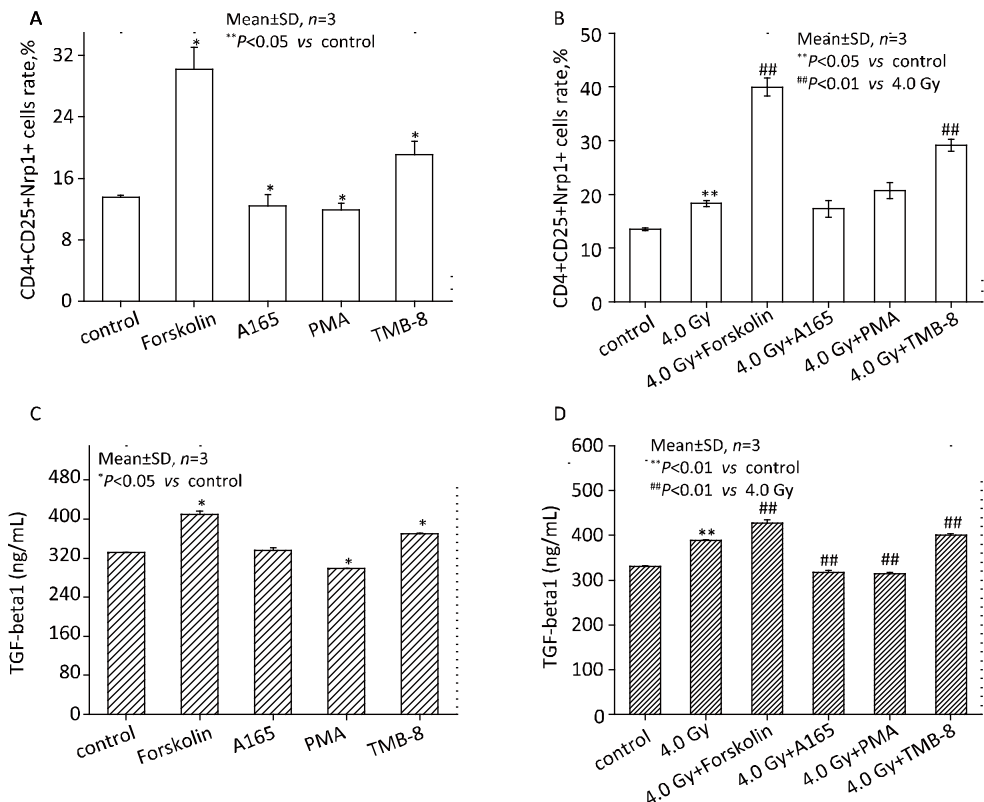


Figure 3. Influence on the production of CD4+CD25+Nrp1+ cells and TGF- β 1 by irradiation and the PLC-PIP2 or AC-cAMP signal pathways. Pharmacologic inhibitors and activators were added 2 h before irradiation. CD4+CD25+Nrp1+ cells in EL-4 cells were detected 48 h after 4.0 Gy X-rays by flow cytometry, the synthesis of TGF- β 1 of EL-4 cells was measured by ELISA. A: the percentage of CD4+CD25+Nrp1+ cells in EL-4 cells, only with pharmacologic; B: the percentage of CD4+CD25+Nrp1+ cells in EL-4 cells, with pharmacologic and 4.0 Gy X-rays; C: the synthesis of TGF- β 1 in EL-4 cells, only with pharmacologic; D: the synthesis of TGF- β 1 in EL-4 cells, with pharmacologic and 4.0 Gy X-rays. (0.12 mg/L of PMA was used to activate the PLC-PIP2 signal pathway; 20 mg/L TMB-8 was used to block the PLC-PIP2 signal pathway; 10 mg/L Forskolin, a transient cAMP inducer, was used to activate the cAMP-PKA signal pathway; 20 mg/L A165, an antagonist of cAMP, was used to block the cAMP-PKA signal pathway).

secretion of TGF- β 1 after 4.0 Gy X rays irradiation, the finding showed that 4.0 Gy X rays significantly induced TGF- β 1 secretion. When the PLC-PIP2 signal pathway was activated or inhibited with TMB-8 or PMA, the secretion of TGF- β 1 was decreased or increased after irradiation. The data also showed that the expression of TGF- β 1 increased in EL-4 cells with Forskolin, and this effect was blocked with A165 (Figure 3D).

DISCUSSION

There is increasing evidence to suggest that radiation can induce immunosuppression^[4-6]. Foxp3 is an established marker of Treg cells^[21]. However, its intra-nuclear localization does not allow it as a marker for Treg selection in subsequent functional analysis. Bruder et al. reported that Nrp1 was strongly and constitutively expressed in murine Treg, and was considered as a reliable surface marker of murine Treg^[7]. Loser et al. showed that Foxp3 could induce Nrp1 expression on CD4+CD25+Treg cells^[22]. A number of previous studies have determined the changes in CD4+CD25+Foxp3+ cells after irradiation. Thus, in the present study, we evaluated the changes in the Nrp1 receptor on CD4+ T cells after irradiation. We found that the Nrp1 receptor was clearly enhanced on CD4+ cells after HDR, and these findings are consistent with those in CD4+CD25+Foxp3+ cells which were up-regulated after 2.0 Gy γ -rays irradiation^[5]. The study reported that Treg cells are more resistant to irradiation than other lymphocytes^[4-6]. These results indicated that Nrp1 may be involved in radioresistance, however, the specific mechanisms need further study. In this report we also found that CD4+CD25+ cell and CD4+CD25+Nrp1+ cell populations showed a good positive correlation in the spleen ($r=0.951$) and thymus ($r=0.969$) (data not shown). This study showed that the cause of immunosuppression after irradiation might be associated with radiation-enhanced CD4+CD25+Nrp1+ cells.

The suppressive mechanisms of Treg cells with multiple modes of action proposed, including mechanisms dependent on cell-cell contact and/or cytokines^[23]. It has been suggested that immunosuppressive cytokines such as TGF- β 1 and IL-10 play an important role in the induction and/or maintenance of regulatory T-cells^[19]. Here we found that TGF- β 1 and IL-10 secretion increased markedly after HDR in thymus and in spleen. This observation is consistent with the finding that 16.0 Gy γ -rays induced and activated the powerful

immunosuppressive cytokine TGF- β 1^[24], which is known to drive Treg cells development^[25]. It is known that IL-10 opposes this function in favor of the differentiation of Th0 cells to the Tr1 type^[26]. It has been shown that IL-10 in the splenocytes was markedly up-regulated after WBI with 2.0 Gy^[27]. Our study also showed that the secretion of IL-10 increased after HDR in spleen and thymus. These results suggested that the mechanism of HDR immune suppression maybe due to the up-regulation of TGF- β 1 and IL-10 which played important roles in Treg cells immune responsiveness control.

It has been well documented that activation of T lymphocytes involves trans-membrane signal transduction via the TCR/CD3 complex coupled to the PLC-PIP2 system^[20]. Perturbation of the TCR/CD3 complex with antigenic or mitogenic stimulation could lead to activation of PLC which causes hydrolysis of PIP2 to form inositol triphosphate (IP3) and diacylglycerol (DG) resulting in mobilization of intracellular calcium and activation of PKC. In addition, the G protein AC system also plays an important role in T cell activation. In the previous study it was demonstrated that low dose X-rays activated the PLC-PIP2 signal pathway and suppressed the cAMP-PKA signal pathway^[28]. Our report also showed that 0.075 Gy X-rays stimulated PKC- α and calcineurin which played important role in the PLC-PIP2 signal pathway, while cAMP and PKA which are important in the cAMP -PKA signal pathway were induced after HDR. These results suggest that CD4+CD25+Nrp1+ cells and TGF- β 1 induced by irradiation might be via activated the cAMP-PKA signal pathway or inhibited the PLC-PIP2 signal pathway. It has been reported that Treg cells can suppress responder T cells in a cAMP-PKA pathway-dependent manner, and it was confirmed that treatment with a cAMP-antagonist enhanced antitumor immune responses^[29]. An increase in cAMP can inhibit IFN- γ secretion^[30]. Moreover, cAMP-elevating agonists, such as PGE2, can also induce Foxp3 gene expression^[31]. A recent study showed that Treg mediates an immediate block of NF- κ B and NFAT signaling in CD4+ T cells through the inhibition of Ca²⁺ signals, which then leads to the suppression of cytokines^[32]. Thus, we next investigated whether the production of CD4+CD25+Nrp1+ cells and TGF- β 1 would change if the PLC-PIP2 and cAMP -PKA signal pathways were activated or inhibited in EL-4 cells. The finding showed that the percentage of CD4+CD25+Nrp1+

cells and TGF- β 1 enhanced markedly with 4.0 Gy X rays, as compared with sham-irradiation, this is consistent with *in vivo*. Interestingly, the Forskolin and TMB-8 could significantly enhance the percentage of CD4+CD25+Nrp1+ cells and TGF- β 1 in EL-4 cells after exposure to ionizing radiation but the production of CD4+CD25+Nrp1+ cells was not blocked with PMA or A165, while the TGF- β 1 was blocked with PMA or A165. This observation is surprising, it suggested that radiation-induced NRP1 +Treg maybe through other signal pathways. It has been reported that PLC-PIP2 and cAMP-PKA signal pathway are involved in the stimulation of thymocytes with low dose irradiation^[28]. These results suggested that Immune-suppressing Effect of HDR could be induced by other signal pathways.

In summary, HDR could induce the production of CD4+CD25+Nrp1+ cells, which inhibit immune responses in general via the release of TGF- β 1 and IL-10. In addition, the PLC-PIP2 and cAMP-PKA signal pathways might be the principal contributors in this process. However, the underlying mechanisms remain to be identified.

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