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Effects of various radiation doses on induced T-helper cell differentiation and related cytokine secretion

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ABSTRACT

Exposure to ionizing radiation often induces T helper (Th) cell differentiation, resulting in an imbalance of Th1 and Th2 cellular subtypes, which can affect the efficacy of cancer radiotherapy. The aim of this study was to analyze differential expression of Th1, Th2 and Th3/Type 1 regulatory T cell (Tr1) subtype–related genes and cytokines in mouse thymocytes after high- and low-dose systemic radiation, using functional classification gene arrays and Elisa assays, and to explore the molecular mechanisms underlying radiation's immune effects and their relationship with Th1/Th2 immunity. We found that expression of 8 genes was upregulated after LDR, while expression of 5 genes was downregulated. After HDR, 54 genes were upregulated and 3 genes were downregulated, including genes related to Th1, Th2 and Th3/Tr1 cellular subtypes, Th1/Th2-type immune response genes and transcription factor–related genes. In the foregoing results, LDR and HDR in the thymus induced opposite patterns of expression for Th1-, Th2- and Th3-type related cytokines *TGF-β*, *C/EBP-β* and *TNF-α*. We also found that expression of Interferon-γ (IFN-γ) and Interleukin-2 (IL-2), which have a moderating effect on immune function, was upregulated after LDR. Furthermore, the secretion of negative regulatory factors Interleukin-1β (IL-1β), Interleukin-4 (IL-4), transforming growth factor-β (TGF-β) and Interleukin-21 (IL-21) was reduced after LDR, but HDR produced the opposite effect and stimulated their expression. These findings suggest that LDR may induce a Th1-type immune response, while HDR may lead to a Th2-type immune response.

Keywords: radiation; dose; Th cells; PCR array; immune response

INTRODUCTION

In recent years, immunological theory has proposed that T helper (Th) cells can be classified according to their secretion of cytokines and that Th functions can be divided into different subgroups composed of Th1, Th2 and Th3/Type 1 regulatory T (Tr1) cells. Th1 cells mainly secrete IL-2 and IFN-γ, enhance the toxic effects of killer T cells, promote macrophage activation and participate in the regulation of cellular immunity, which facilitates the differentiation of cytotoxic T cells and mediates the cellular immune hypersensitive response. Th2 cells secrete IL-4, IL-6 and IL-10, which mediate humoral immunity and promote antibody production [1, 2]. Th3/Tr1 cells secrete TGF-β, which exerts a negative regulatory effect on immune responses. The balance of Th1/Th2 cells is important for maintaining immune health of the host [3]. Th1 cells promote resistance to intracellular pathogens and secrete cytokines such as IFN-γ, a major cytokine associated with host resistance to viral infections. Th2 cells promote resistance to extracellular pathogens and importantly to the current study, antagonize the production and activity of Th1 cytokines, including IFN-γ [4, 5]. Thus, an altered Th1/Th2 balance can influence host susceptibility to a

variety of immune-mediated diseases, including allergy, autoimmunity and increased infections [6, 7].

Exposure to ionizing radiation often reduces Th1 function, resulting in a Th1/Th2 imbalance that may affect the efficacy of cancer radiotherapy [8, 9]. It has been reported that ionizing radiation induced a lasting Th2 immune response while depressing Th1 immune responses in experimental animals and atomic bomb survivors. Th1/Th2 imbalance also accounts for radiation-induced immunosuppression [10–12]. A large number of studies have confirmed that low-dose radiation (LDR, 0.075–0.2 Gy) and high-dose radiation (HDR, 2.0–6.0 Gy) induce different biological effects that can stimulate organism or tissue adaptive functions, expressed as a series of adaptive reactions [13], activation of immune function [14, 15] and prevention and treatment of inflammatory diseases [16], in which the activation of T lymphocytes plays a central role in the radiation-induced immune effect. In this study, we used Th1-Th2-Th3 functional classification arrays to detect and analyze HDR- and LDR-induced differential expression of thymus Th cell subtype function-related genes, and enzyme-linked immunosorbent assay (ELISA) was used to verify the expression of cytokines related to immune functional regulation, in order to explore the molecular mechanisms underlying the effect of different radiation doses on immune function.

MATERIALS AND METHODS

Experimental animals and groups

A total of 16 healthy ICR mice, half male and half female, 6 to 8-week-old, weighing 18–22 g, were purchased from Jilin University Bethune Medical Laboratory Animal Center (Changchun, China), production license number SCXK-Kyrgyzstan 2008-0005 and use of permits lot number SCXK (Jilin) 2008-0011. Mice were housed in individual cages in a temperature-controlled room at 22 ± 2°C with relative humidity of 50 ± 10% and a 12 h dark/light cycle. Food and water were provided *ad libitum* throughout the experiment. Mice were irradiated in a holder designed to immobilize unanesthetized mice to ensure the accuracy of radiation doses. The mice were randomly divided into three groups: control group ($n = 4$), LDR group ($n = 4$) and HDR group ($n = 8$).

Irradiation conditions

Irradiation was performed with an X-ray fixed deep treatment machine X.S.S.205 FZ (Dandong, China). HDR group mice were whole-body irradiated at a single high dose (2.0 Gy) of X-rays administered from a source–skin distance of 60 cm, at the dose rate of 343 mGy/min. LDR group mice were whole-body irradiated at a single low dose (0.075 Gy) of X-rays administered from a source–skin distance of 178.50 cm, at a dose rate of 12.5 mGy/min.

Tissue collection

The mice were euthanatized 24 h after X-ray irradiation, and the thymus tissue of each mice was individually labeled and collected in Eppendorf tubes then stored in liquid nitrogen. Dry ice was used the next day to transport the tissues to the test facility (KangCheng Biological, Shanghai, China). The tissues were homogenized using Teflon tissue homogenizers (BILON-08; Shanghai Bilon Instrument Manufacturing Co. Ltd, Shanghai,China) as recommended by the manufacturer. The resulting single-cell suspensions were centrifuged for 10 min at 12 000 r/min and the cytokines levels were measured in the homogenates by ELISA (all from R&D Systems).

RNA preparation

Total RNA was isolated from tissue samples with the use of TRIZOL® Reagent (Invitrogen,USA) and an RNeasy Mini Kit (Qiagen,Germany) according to the manufacturer's instructions. The RNA was treated with an RNase-free DNase I Kit (Qiagen) to remove contaminating genomic DNA. RNA quality was checked with 1% agarose gel electrophoresis, and RNA integrity was analyzed with a 2100 Bioanalyzer (Agilent Technologies,USA). RNA quantity was determined with the ND-1000 Spectrophotometer (Nanodrop Technology,Wilmington, DE). Samples with 260/280 nm adsorbance ratio of >1.8–2.0 were used for further experiments.

Gene profiling microarray

Total RNA (1 μg) was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Gene expression was analyzed with the Mouse Th1-Th2-Th3 RT^2 Profiler™ PCR Array (Qiagen). cDNA was amplified in the presence of 84 specific primers coated in 96-well microtiter plates on a CFX96 Real-Time System (Bio-Rad, Munich, Germany). The 84 evaluated genes are associated with T cell–derived cytokines, CD4+ T cell markers, the T regulatory (Treg) cell network, T cell activation and relevant transcription factors. Samples were normalized to *GAPDH* as the reference gene. CFX Manager software (Bio-Rad,USA) was used to view the amplification of each gene and corresponding Ct values.

Enzyme-linked immunosorbent assay

ELISA was used to quantify concentrations of Th1-Th2-Th3-related cytokines IFNγ, IL-2, IL-1β, IL-4, TGF-β and IL-21 in each group. ELISA kits for the detection of each cytokine were obtained from R&D (R&D Systems, Minneapolis, MN). The assays were performed in duplicate with 50 μl of sample added to each well following the manufacturer's instructions. The readings were taken in an Epoch BioTek® ELX 800 plate reader (BioTek, Winooski, VT). The OD was read at 450 nm with reference to 630 nm. A standard curve was prepared for each cytokine, and the corresponding curve formulas were used to calculate the sample concentrations.

Statistical analysis

The relative expression of each gene in the two dose groups was calculated by the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates the fractional cycle number when the fluorescent signal reaches detection threshold. The relative amount of the control was calculated and compared with the relative amount of the sample (quantitative ratio). The ratio of the irradiated group to the sham group was >2.0, and the decrease was <0.5 [17]. The data for quantitative gene expression were characterized using descriptive statistics, and the statistical significance of differences in gene and cytokine expression between groups was calculated using Student's *t*-test. All statistical tests were

performed with SPSS version 22.0 (SPSS, Inc., Chicago, USA). $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Gene expression profile of Th1-Th2-Th3 cytokine-related genes after various doses of radiation

The expression of differentially expressed genes in the Th1-Th2-Th3 RT^2 Profiler™ PCR Array was analyzed. The LDR group had 13 differentially expressed genes compared with the control group, while the HDR group had 57 differentially expressed genes compared with controls. Among the differentially expressed genes, 8 genes were significantly upregulated in mice thymocytes after LDR and 5 genes were significantly downregulated ($P < 0.05$). After HDR, 54 genes were upregulated in thymocytes and 3 genes were downregulated (Fig. 1).

LDR-induced Th1-Th2-Th3-related gene functional analysis

Table 1 shows that LDR induced upregulation of Th1 cell–related genes *Stat4* and *Socs1*, downregulation of Th2 and Th3/Tr type cell–associated genes *Il-4ra, Cebpb, Gata3* and *Tgfb3* and upregulation of the Th1-type immune response gene *Sftpd*.

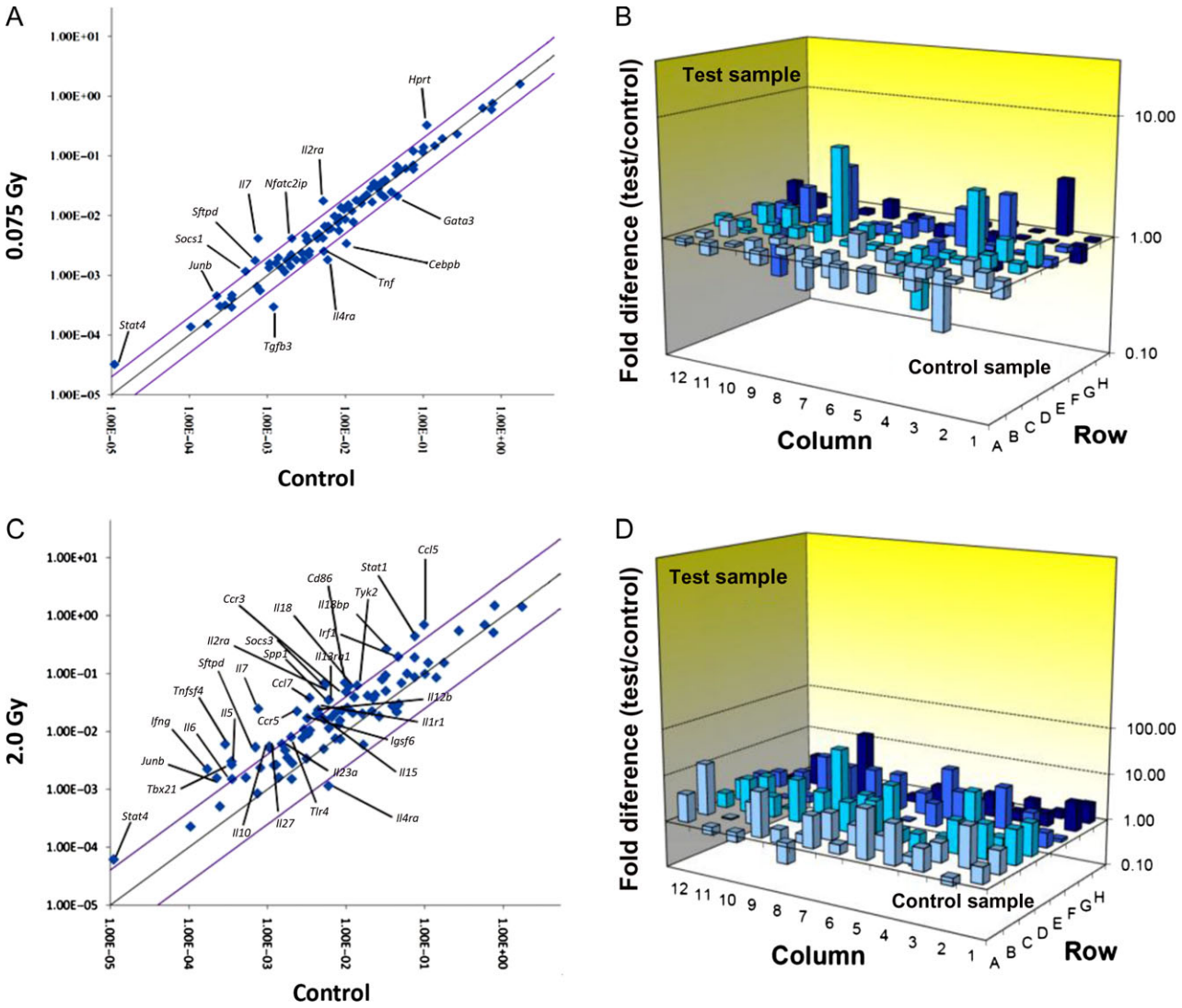

Fig. 1. Effect of LDR and HDR on Th1-Th2-Th3-related genes in thymus cells. (A) Scatter-plot of gene expression in the LDR group. The genes above the top line and below the bottom line displayed a >2.0-fold change in expression between the LDR and sham-control group. (B) Fold differences with respect to control tissue of differentially expressed genes in the LDR group. (C) Scatter-plot of gene expression in the HDR group. The genes above the top line and below the bottom line displayed a >2.0-fold change in expression between the HDR and sham-control group. (D) Fold differences with respect to control tissue of differentially expressed genes in the HDR group.

Table 1. LDR-induced differential expression of *Th1-Th2-Th3*-related genes in the thymus

Category	Gene symbol	Gene name	Fold change	*P* value
Th1 cell–related genes				
Upregulated	*Stat4*	Signal transduction and transcriptional activator 4	2.97	0.0000
	Socs1	Suppressor of cytokine signaling 1	2.22	0.0012
Downregulated	*Tnf*	Tumor necrosis factor	−2.09	0.0025
Th2 cell–related genes				
Upregulated	*none*			
Downregulated	*Il-4Rα*	Interleukin 4 receptor, α	−3.25	0.0018
	Cebpb	cAMP reaction element binding protein	−3.02	0.0034
	Gata3	GATA binding protein 3	−2.20	0.0213
Th3/Tr1 cell–related genes				
Upregulated	*none*			
Downregulated	*Tgfb3*	Transforming growth factor, beta 3	−4.03	0.0012
Th1 type immune response genes				
Upregulated	*Sftpd*	Surfactant-related protein D	2.54	0.0018
Downregulated	*none*			

HDR-induced Th1-Th2-Th3-related gene functional analysis

A 2.0 Gy dose of radiation induced upregulation of Th1 type, Th2 type and Th3/Tr type cell–related genes, but had no effect on Th1-type immune response–related gene expression (Table 2). In contrast, Th2-type immune response–related genes *Cd86, Il-18, Il-10* and *Irf4* were significantly upregulated.

Analysis of transcription factors and related genes in Th1-Th2-Th3 cells induced by various doses of radiation

LDR induced upregulation of *Stat4* and downregulation of *Cebpb* and *Gata3* (Table 3). HDR induced significant upregulation of transcription factor–related genes *Tbx21, Stat1, Stat4, Irf1, Nfkb1* and *Cebpb*, but no genes were significantly downregulated by HDR.

LDR and HDR induced opposing effects on Th1-Th2-Th3-related genes

Fig. 2 shows that after high and low doses of X-ray irradiation in mice, the thymus cells expressed opposing patterns of response for three Th cell–related genes. *TGF-β, C/EBP-β* and *TNFα* were significantly upregulated more than 2-fold after HDR irradiation, but were downregulated after LDR irradiation, with the decrease in *TGF-β* expression being >4-fold ($P < 0.05$).

Th1-Th2-Th3-related cytokine expression in mouse thymocytes after various doses of radiation

In final experiments, we determined the effect of LDR and HDR on cytokine secretion from Th1-Th2-Th3 cells in the mouse thymus. Figure 3 shows that IFN-γ and IL-2 secretion were significantly upregulated after LDR ($P < 0.05$), while IL-4 ($P < 0.05$), IL-1β, IL-21 and TGF-β ($P < 0.01$) were downregulated. In contrast, regulation of cytokine secretion after HDR showed the opposite pattern.

DISCUSSION

The thymus is an important central immune organ for T lymphocyte differentiation and maturation, and is one of the target organs highly sensitive to ionizing radiation. Therefore, the radiation response of thymocytes is an important focus of radiation immunology research. Different doses of ionizing radiation can produce different biological effects on the body. Higher doses of irradiation have a detrimental effect on the body, while lower doses of ionizing radiation may have the opposite effect. The United Nations Atomic Radiation Effects Scientific Committee reported in 1986 that the level of radiation should be considered low when >0.05 Gy of high–Linear Energy Transfer (LET) radiation or <0.2 Gy of low-LET radiation, while radiation dose rates should be <0.05 mGy/min to be considered low. A large number of experimental studies have confirmed that low doses of ionizing radiation can stimulate immune function [18, 19].

Table 2. HDR-induced differential expression of *Th1-Th2-Th3*-related genes in the thymus

Category	Gene symbol	Gene name	Fold change	*P* value
Th1 cell–related genes				
Upregulated	*Ifng*	Interferon γ	13.14	0.0022
	Il-2ra	Interleukin 2 receptor, α	13.00	0.0052
	Ccr5	Chemokine receptor 5	9.51	0.0024
	Il-18bp	Interleukin 18 binding protein	8.24	0.0324
	Tbx21	T-box 21	7.68	0.0003
	Stat1	Signal transduction and transcriptional activator 1	5.96	0.0007
	Il-18	Interleukin 18	5.63	0.0109
	Stat4	Signal transduction and transcriptional activator 4	5.61	0.0001
	Igsf6	Immunoglobulin superfamily member 6	5.34	0.0032
	Il-12b	Interleukin 12B	4.83	0.0042
	Irf1	Interferon regulatory factor 1	4.28	0.0458
	Socs1	Suppressor of cytokine signaling 1	3.03	0.0005
	Tnf	Tumor necrosis factor	3.00	0.0053
	Socs5	Suppressor of cytokine signaling 5	2.89	0.0074
	Il-18r1	Interleukin 18 receptor 1	2.77	0.0033
	Csf2	Colony stimulating factor 2	2.07	0.0013
	Il-2	Interleukin 2	2.06	0.0002
Downregulated	*none*			
Th2 cell–related genes				
Upregulated	*Ccl7*	Chemokine ligand 7	11.14	0.0034
	Il-5	Interleukin 5	8.74	0.0003
	Ccl5	Chemokine ligand 5	7.17	0.0098
	Il-13Ra1	Interleukin 13 receptor, α1	5.88	0.0061
	Il-10	Interleukin 10	5.27	0.0011
	Ccr3	Chemokine receptor 3	4.87	0.0101
	Tlr4	Toll receptor 4	4.06	0.0021
	Maf	Transonic fibrosarcoma AS42 oncogene homologues	3.11	0.0126
	Irf4	Interferon regulatory factor 4	2.80	0.0028
	Cebpb	cAMP reaction element binding protein	2.51	0.0103
	Ccl11	Chemokine ligand 11	2.18	0.0316
	Jak1	Janus kinase 1	2.02	0.0027

Continued

Table 2. Continued

Category	Gene symbol	Gene name	Fold change	*P* value
Downregulated	*Il-4ra*	Interleukin receptor 4, α	–5.17	0.0059
	Ccr4	Chemokine receptor 4	–2.79	0.0165
	Gfi1	Independent growth factor 1	–2.02	0.0041
Th3/Tr1 cell–related genes				
Upregulated	*Il-23a*	Interleukin 23, α	4.03	0.0015
	Il-17a	Interleukin 17A	2.18	0.0001
	Tgfb3	Transforming growth factor, β3	2.18	0.0012
Downregulated	*none*			
Th2 type immune response genes				
Upregulated	*Cd86*	CD86 antigen	7.26	0.0096
	Il-18	Interleukin 18	5.63	0.0109
	Il-10	Interleukin 10	5.27	0.0011
	Irf4	Interferon regulatory factor 4	2.80	0.0028
Downregulated	*none*			

Table 3. Effects of LDR and HDR on *Th1-Th2-Th3* cell–related transcription factors in mouse thymus

Features	Gene symbol	Brief description of genes	Fold change	*P* value
LDR				
Upregulated	*Stat4*	Signal transduction and transcriptional activator 4	2.97	0.0001
Downregulated	*Cebpb*	cAMP reaction element binding protein	−3.02	0.0034
	Gata3	GATA binding protein 3	−2.20	0.0213
HDR				
Upregulated	*Tbx21*	T-box 21	7.68	0.0003
	Stat1	Signal transduction and transcriptional activator 1	5.96	0.0007
	Stat4	Signal transduction and transcriptional activator 4	5.61	0.0001
	Irf1	Interferon regulatory factor 1	4.28	0.0458
	Nfkb1	The light peptide gene enhances the nuclear factor 1, p105	2.79	0.0292
	Cebpb	cAMP reaction element binding protein	2.51	0.0103
Downregulated	*none*			

The functional classification chip, because of its strong targeting, avoids the interference of the irrelevant genes in the spectrum chip, and has high accuracy and sensitivity. Therefore, it has many applications in the study of radiation immunology. The latest development is the PCR functional classification chip, using real-time quantitative PCR technology and gene chip technology to provide accurate quantitative detection of mRNA expression levels for hundreds of genes on the same chip [20]. The chip technique overcomes the shortcomings of the previously applied differential display PCR method, including high false positivity rates, poor reproducibility and bias for high-copy genes. T helper (Th) cell subtypes Th1 and Th2 originate from common naïve precursor cells in response to antigen and cytokine stimulation. Th cells are functionally divided according to their cytokine profiles and types of mediated immune reaction. It is well recognized that disturbances in the balance between Th1 and Th2 responses can promote immune-mediated diseases. An enhanced Th2 response is involved in atopic diseases such as asthma, whereas a dominating Th1 response is implicated in chronic autoimmune diseases like type 1 diabetes and rheumatoid arthritis.

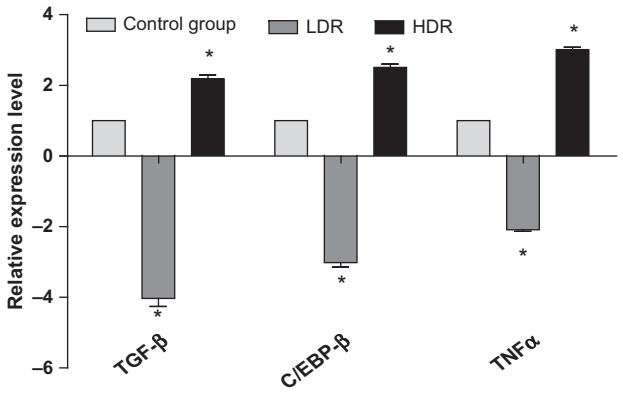

Fig. 2. LDR- and HDR-induced expression of *Th1-Th2-Th3*-related genes. Gene expression was downregulated after LDR and upregulated in the HDR group. Gene expression differences were significant with fold changes >2.0; this is an additional criterium of biological relevance. (*$P < 0.05$ vs control group).

In this study, the results using a Th1-Th2-Th3 functional sorting chip showed that LDR mainly upregulated *Stat4* in Th1 cells and downregulated the key transcription factor *Gata3* in Th2 cells; meanwhile, expression of *TGF-β* was strongly suppressed in Th3/Tr1 cells. *TGF-β* has now been determined to be the sole factor exerting anti-proliferative effects on Th1 and Th2 cells that eventually lead to the Th3 immune response, by perturbing the differentiation of either Th1 or Th2 cells and inhibiting proinflammatory cytokine production [21]. It has been reported that *IFN-γ/IL-4* and *T-bet/GATA3* play a key role in the regulation of Th1/Th2 immune function [22]. The finding by Gridley *et al.* that alterations in CD4+ T cell gene expression, although strikingly different in 0.01 Gy- and 0.10 Gy-exposed cells, supports a shift in the Th1/Th2 balance toward the Th1 phenotype and indicates that LDR radiation promotes Th1 cell differentiation. This suggests that cell-mediated responses against virus-infected and other aberrant cells can be enhanced [23], which may induce Th1 immune responses and ultimately improve immune function. Recent studies have shown that IL-12 can differentiate $CD4^+$ cells into Th1 cells via the Stat4 signaling pathway, whereas IL-4 induces the differentiation of naïve Th0 cells into Th2 cells through the Stat6 signaling pathway. The polarization of Th1/Th2 cells is related to the expression of different Socs members [24]. Socs proteins are negative regulators of the Jak–Stat signal transduction pathway that is triggered by many cytokine–receptor interactions [25]. The expression of *Socs1* was twice as high when Th cells were differentiated into Th1 cells. When Th cells were differentiated into Th2 cells, the expression of *Socs1* was 23 times higher than that of Th1 cells [26]. In the present study, it was found that the expression of *Socs3* was two times

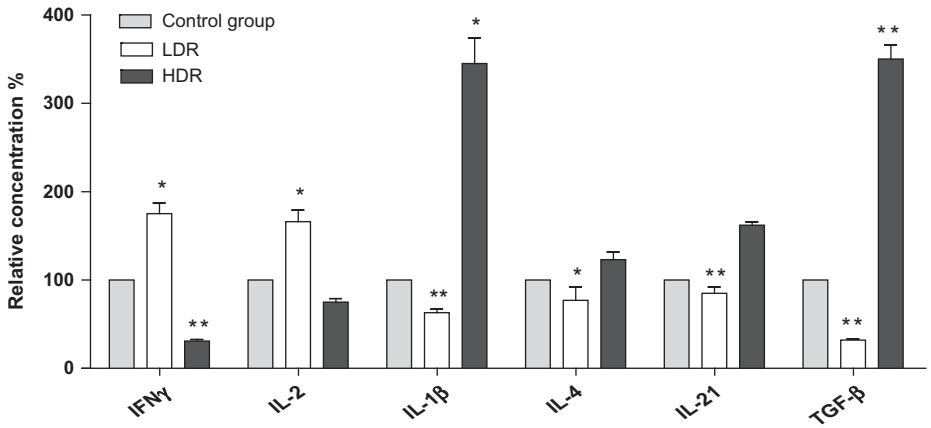

Fig. 3. Effects of LDR and HDR on *Th1-Th2-Th3*-related cytokine secretion by mouse thymocytes. IFN-γ, IL-2, IL-1β, IL-4, IL-21 and TGF-β concentrations in culture supernatants were determined by ELISA. Mean values of triplicate cultures were obtained for each of three experiments, and results were expressed as relative expression level (%) compared with the control group (*$P < 0.05$, **$P < 0.01$).

greater than that in the sham group after 2.0 Gy irradiation, while *Socs1* expression was 3.03 times greater, indicating that high-dose irradiation may upregulate *Socs3* and induce differentiation of Th2 cells.

IL-1 is a macrophage-derived pro-inflammatory cytokine that assists in T cell activation. The receptor for IL-1 plays an important role in inflammation by activating nuclear factor kappa B and neuromodulation in the central nervous system[27, 28]. *Tbx21* (*T-box 21*, also known as *T-bet/Tbx21*) was the other gene showing significant upregulation after 0.1 Gy exposure. This gene encodes a transcription factor that activates *IFN-γ* production in the Th1 subset and promotes development of Th1 cells while inhibiting Th2 cells[29, 30]. It is also well known that TGF-β1 is a negative immunoregulatory factor secreted by Treg cells. Its main function is to inhibit the proliferation of T cells and the secretion of inflammatory cytokines, and it plays an important role in maintaining immune tolerance and immune balance [31]. In addition, the results of this study indicated that the HDR-upregulated genes were positively regulated cytokines (IL-12b, IL-15, IL-18, GM-CSF, etc.), negatively regulated cytokines (IL-5, TGF-β, IL-10, IL-6, IL-17, IL-23, etc.) and negative regulatory co-stimulatory molecules (CTLA-4). Furthermore, HDR downregulated the early effect of the *Gfi-1* and *CD124* (*IL-4ra*) genes in Th2 cells, so that T cells mainly regulated Th3/Tr1 cell differentiation and induced Th2 cells to inhibit the immune response. At the same time, we found that the cytokines related to Th1-Th2-Th3 cells in the thymus of mice that were most affected by high- and low-dose radiation were TGF-β, C/EBP-β and TNFα. We further confirmed the expression of Th1-Th2-Th3 cell–related cytokines with ELISA. IFNγ and IL-2 secretion were positively regulated by LDR irradiation ($P < 0.05$), which had a negative effect on IL-1, IL-4, TGF-β and IL-21 expression, and there was a significant difference between the LDR and HDR groups, with the HDR group showing an opposite pattern of expression o that of the LDR group.

In summary, LDR and HDR induced different immune effects. LDR induced the Th1 immune response to increase immune function, while HDR induced Th3/Tr1 cells and inhibited the immune response. These experimental results will add a new experimental basis for radiological immunology theory.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

FUNDING

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