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Exacerbation of autoimmune thyroiditis by a single low-dose of whole-body irradiation in non-obese diabetic-\(H2^{b4}\) mice

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Running title: Irradiation and thyroid autoimmunity

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Key words: thyroid autoimmunity; thyrotropin receptor; thyroglobulin; adenovirus; iodide
Abstract

**Purpose:** To evaluate how irradiation affects thyroid autoimmunity in mouse models of Hashimoto’s thyroiditis and Graves’ hyperthyroidism.

**Materials and methods:** Non-obese diabetic (NOD)-H2<sup>bd</sup> mice spontaneously develop anti-thyroglobulin (Tg) antibodies and thyroiditis when supplied with sodium iodine (NaI) in the drinking water. BALB/c mice develop anti-thyrotropin receptor (TSHR) antibodies and hyperthyroidism following immunization with adenovirus expressing TSHR (Ad-TSHR). Mice were irradiated as follows; a single whole-body irradiation with 0.05, 0.5 or 3 Gy one week before or after the beginning of NaI or immunization with Ad-TSHR, fractionated whole-body irradiations with 0.05 Gy twice a week or 0.5 Gy once a week from one week before NaI or Ad-TSHR immunization, or a single regional irradiation to the thyroid gland with 0.5 Gy one week before NaI. The effect of a single irradiation with 0.05, 0.5 or 3 Gy on splenocytes was also evaluated.

**Results:** A single whole-body irradiation with 0.5 Gy one week before NaI exacerbated thyroiditis and increased anti-Tg antibody titers in NOD-H2<sup>bd</sup> mice. In contrast, any irradiation protocols employed did not affect incidence of hyperthyroidism or anti-TSHR antibody titers in BALB/c mice. High-dose irradiation increased the relative ratios of effector T cells to regulatory T cells (an indication of enhanced immune status) but kills most of T cells.

**Conclusions:** These results indicate that a single whole-body low-dose irradiation with
0.5 Gy exacerbates thyroiditis in NOD-H2\textsuperscript{h4} mice, data consistent with some clinical evidence for increased incidence of thyroid autoimmunity by environmental irradiation.
Introduction

Despite the definitive harmful effects of high-dose irradiation such as carcinogenic action and systemic immune suppression, low-dose irradiation is reported to have some stimulatory effects on living organisms. These effects, called “radiation hormesis”, include stimulation of growth rate (Luckey 1982), enhancement of survival after lethal high-dose of irradiation (Yonezawa et al. 1990), prolongation of life span (Ducoff 1975) and elevation of resistance to oxygen toxicity (Lee & Ducoff 1984). Numerous articles also demonstrated “beneficial” effects of the low-dose irradiation on immune system in humans and animals (Safwat 2000). For examples, low-dose irradiation on the one hand enhances tumor immunity (Ina et al. 2005, Kojima et al. 2004, Hong-Sheng et al. 2004, Hashimoto et al. 1999, Hosoi & Sakomoto 1993) while on the other hand suppresses autoimmune responses (James et al. 1990, Takahashi et al. 2000, Ootsuyama et al. 2003, Ina & Sakai 2004, Tanaka et al. 2005) in experimental animal models. These apparently opposing (suppressing versus enhancing) effects nonetheless can cure various diseases.

Several lines of clinical and experimental evidence indicate that the thyroid gland is one of the most vulnerable organs by irradiation, particularly in childhood. Indeed an increased risk of thyroid neoplasia has been confirmed in cohorts exposed to environmental irradiation in Nagasaki and Hiroshima, the Marshall Islands, the Nevada nuclear test site and Chernobyl (Ehamen et al. 2003, Cardis et al. 2006). However, relationship between irradiation and thyroid autoimmunity remains controversial. Thus significant correlations between irradiation and thyroid autoimmunity were observed in studies conducted in Chernobyl (Vermigilio et al. 1995, Vykhovanets et al.
1997, Lomat et al. 1997, Pacini et al. 1998), but not in those in the Marshall Islands and the Nevada test site (Kerbers et al. 1993, Cronkite et al. 1995, Cronkite et al. 1995, Takahashi et al. 1999). In addition, one (Nagataki et al. 1994), but not other (Morimoto et al. 1987, Yoshimoto et al. 1995), survey of the atomic bomb survivors in Nagasaki and Hiroshima supports the significant relationship. It should be noted here that the diagnostic criteria of thyroid autoimmunity is not identical among these studies.

Autoimmune thyroid diseases include Hashimoto’s thyroiditis and Graves’ disease, both being common organ-specific autoimmune diseases in humans. The former is characterized by destruction of the thyroid gland by cellular immune response and consequent hypothyroidism, and the latter by overstimulation of the thyroid gland by stimulatory anti-thyrotropin receptor (TSHR) autoantibodies (e.g., humoral immune response) and hyperthyroidism (Braverman & Utiger. 2000). Since animal models of both diseases can now be available, the present study was designed to evaluate the effect of irradiation on animal models of Hashimoto’s thyroiditis and Graves’ disease. Among several animal models of Hashimoto’s thyroiditis and Graves’ disease available, we used in this study a spontaneous model of NOD-H2<sup>hi</sup> mice for Hashimoto’s thyroiditis (Rasooly et al. 1996, Podolin et al. 1993) and an induced model of Graves’ disease using recombinant adenovirus expressing the TSHR (Nagayama et al. 2002, Chen et al. 2003).
Materials and methods

Mice

Female BALB/c (6 to 7 weeks old) mice were purchased from Charles River Japan Laboratory Inc. (Tokyo, Japan). NOD-H2^b^ mice were obtained from Jackson Laboratory Inc. (Bar Harbor, ME, USA) and bred in the animal facility at Nagasaki University; both male and female mice were used for the current study. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. All the mice were kept in a specific pathogen free condition.

Graves' model: induction of Graves' hyperthyroidism by immunization with adenovirus coding the TSHR

Construction, amplification and purification of non-replicative recombinant human adenovirus expressing the human TSHR-A subunit (AdTSHR289, kindly provided by Drs. Sandra M. McLachlan and Basil Rapoport) and determination of the viral particle concentration were described previously (Nagayama et al. 2002, Chen et al. 2003). Mice were injected intramuscularly in the quadriceps with 100 μl phosphate buffered saline (PBS) containing 10^{10} particles of AdTSHR289 on two occasions at three-week-intervals. Blood and thyroid tissues were obtained two weeks after the final immunization.

Thyroxine (T_4) and anti-TSHR antibody measurements
Serum free T₄ concentrations were measured with a radioimmunoassay kit (DPC free T₄ kit; Diagnostic Products, Los Angeles, CA, USA). The normal range was defined as the mean ± 3 standard deviation (S.D.) of control untreated mice.

Anti-TSHR antibodies in mouse sera were determined by flow cytometry assay with Chinese hamster ovary (CHO) cells stably expressing the full-length TSHR (~2 x 10⁶ receptors/cells), mouse sera (1:100 dilution) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) as previously described (Mizutori et al. 2006). The titers were expressed as mean fluorescence intensities (MFI).

Hashimoto’s model: induction of thyroiditis by sodium iodine (NaI)

NOD-H₂h₄ mice (6 to 7 weeks old) were supplied with 0.05 % NaI in the drinking water for eight weeks, and then blood and thyroid tissues were obtained.

Evaluation of thyroiditis

Thyroid tissues were removed, and fixed in 10 % formalin in PBS. Tissues were then embedded in paraffin, and 5-μm-thick sections were prepared and stained with hematoxylin-eosin. Thyroiditis was assessed for extent of lymphocyte infiltration as previously described (Allen et al. 1986); briefly, grade 0, normal thyroid; grade 1, less than 10 % lymphocytic infiltration of the thyroid; grade 2, 10 to 30 % lymphocytic infiltration; grade 3, 30 to 50 % lymphocytic infiltration; grade 4, greater than 50 % lymphocytic infiltration. The final thyroiditis scores were expressed as means of at least three noncontiguous sections from each thyroid gland.
**Enzyme-linked immunosorbent assay (ELISA) assay for anti-thyroglobulin (Tg) antibody measurements**

Mouse Tg was purified from mouse thyroid glands as previously described (Saboori et al. 1993). ELISA wells were coated overnight with 100 μl Tg protein (10 μg/ml) and incubated with mouse sera (1:300 dilution). After incubation with horseradish peroxidase conjugated anti-mouse IgG, IgG1 or IgG2b (Sigma-Aldrich Corp.), color was developed using orthophenylene diamine and H₂O₂ as substrate and optical density read at 492 nm.

**Irradiation protocol**

Anesthetized mice were exposed to either a single (0.05, 0.5 or 3 Gy/mouse) or fractionated (0.05 Gy twice a week or 0.5 Gy once a week for five weeks in Graves’ model and nine weeks in Hashimoto’s model) doses of γ-irradiation with an EXS-300 γ-irradiator (200 kV; 15 mA; filter, 0.5 mm aluminum and 0.5 mm copper; 0.47 Gy/min; Toshiba, Tokyo, Japan). Local irradiation to the thyroid gland was performed by shielding non-thyroidal area with lead collimators.

**Flow cytometry**

The cells were stained with FITC or phycoerythrin (PE)-conjugated anti-anti-cluster of differentiation 4 (CD4) (H129.19), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD25 (7D4) or anti-Foxp3 (FJK-16s; Foxp3 staining kit) (PharMingen, San Diego, CA, USA or eBioscience, San Diego, CA, USA) according to the manufactures’ instructions, and analyzed on a FACScan flow cytometry using CellQuest software (BD Biosciences).
**Statistical analysis**

Levels of T₄, antibodies and cytokines, and thyroiditis scores were analyzed by t-test, incidences of hyperthyroidism by chi-square test and correlation coefficient by linear regression analysis. A ‘p’ value of less than 0.05 was considered statistically significant.
Results

The effect of irradiation on Hashimoto’s model

NOD-H2\textsuperscript{b4} mice supplied with NaI in the drinking water for eight weeks frequently developed Hashimoto’s thyroiditis with thyroiditis scores of 1.718 ± 1.427 (mean ± S.D.) (Fig. 1A). The representative histology of the thyroid glands is shown in Fig. 2; naive mice showed little intrathyroidal lymphocyte infiltration (grade 0, Fig. 2A), while NaI-treated mice developed mild to severe thyroiditis (for examples, grade 2 thyroiditis in Fig. 2B and grade 4 thyroiditis in Fig. 2C). In these mice, development of thyroiditis was accompanied by appearance of anti-Tg autoantibodies in sera with the titers of 0.219 ± 0.262 (Fig. 1B). There is a weak but significant correlation between thyroiditis scores and anti-Tg antibody titers (r = 0.60, p < 0.05; data not shown).

A single dose of whole-body irradiation with 0.5 Gy one week prior to the beginning of NaI significantly enhanced the severity of thyroiditis (3.179 ± 1.175, p < 0.01) and the titers of anti-Tg autoantibodies (0.409 ± 0.262, p < 0.05). However, the ratios of IgG1/IgG2b (e.g., T helper type 2 (Th2)/Th1) were unchanged (data not shown). In contrast, none of other irradiation protocols mentioned in the Materials and methods did not affect the degree of thyroiditis or the titers of anti-Tg autoantibodies (Fig. 1).

Next experiments were conducted to clarify (i) whether whole-body irradiation is critical for the aforementioned irradiation effects or local irradiation to the thyroid gland is sufficient, and (ii) whether or not adaptive response would be observed in this model. Adoptive response is defined by the induction of radioresistance to subsequent higher doses of radiation by pretreatment with low radiation doses (Yonezawa et al. 1990). Groups of mice were first exposed to very low dose of whole-body irradiation (0.05 Gy)
and one or two weeks later challenged with 0.5 Gy whole-body irradiation. In another
group of mice, irradiation exposure was restricted to the thyroid glands. As shown in
Fig. 3, thyroiditis was enhanced in all the groups of mice that received 0.5 Gy
whole-body irradiation with/without pretreatment with a very low dose (0.05 Gy)
irradiation one or two weeks before. Further, regional irradiation to the thyroid gland
failed to significantly enhance thyroiditis scores. Thus these data demonstrate lack of
adaptive response and importance of whole-body irradiation in irradiation-induced
exacerbation of thyroiditis.

The effect of irradiation on Graves’ model

We have recently established a novel mouse model of Graves’ hyperthyroidism
using adenovirus expressing the TSHR-A subunit (AdTSHR289) (Nagayama et al. 2002,
Chen et al. 2003). Thus susceptible BALB/c mice injected with AdTSHR289
exhibited increased serum thyroid hormone concentrations (thyroxine, T4) and
anti-TSHR antibody titers (Fig. 4). The representative histology of the thyroid glands
from hyperthyroid mice is depicted in Fig. 5, showing hyperplasia and hypertrophy of
the thyroid follicular epithelial cells, findings comparable with those seen in the
overstimulated thyroid gland.

However, none of irradiation protocols described in the Materials and methods
altered serum T4 levels or anti-TSHR antibody titers as shown in Fig. 4.

The effect of irradiation on immune cells

We also studied the effect of irradiation on immune cells (splenocytes). The
numbers of the total spleen cells, the percentages of CD4+ T cells, CD8+ T cells, CD19+

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B cells, CD4+CD25+ T cells and CD4+Foxp3+ T cells were determined, and the ratios of CD4+CD25+ T cells or CD4+Foxp3+ T cells to CD4+ T cells were calculated (Figs. 6 and 7). Splenocytes were prepared three to four days after irradiation. A single dose of whole-body irradiation with 0.05 or 0.5 Gy had little effect on spleen cells, except a slight decrease in the numbers of splenocytes by 0.5 Gy (approximately 30%). However, 3 Gy irradiation significantly decreased the number of splenocytes and the percentage of CD8+ T cells, while increased the percentages of CD4+ T cells, CD4+CD25+ T cells and CD4+Foxp3+ T cells, and the ratios of CD4+CD25+ T cells to CD4+ T cells (not the ratios of CD4+Foxp3+ T cells to CD4+ T cells). Although CD25 is originally reported to be expressed on naturally-occurring regulatory T cells (Treg) (Sakaguchi et al. 1995), recent studies revealed that CD25 is also expressed on activated effector T cells (Teff) and that Foxp3, a transcriptional factor, is a highly specific marker for Treg (see Sakaguchi. 2005 for a review). Therefore, an increase in the ratios of CD4+CD25+ T cells to CD4+ T cells, not the ratios of CD4+Foxp3+ T cells to CD4+ T cells, indicates a relative increase in the ratios of Teff to Treg.
Discussion

The present studies were performed to evaluate the effect of irradiation on thyroid autoimmunity by using mouse models of Hashimoto’s thyroiditis and Graves’ hyperthyroidism, because relationship between irradiation (particularly low-dose) and thyroid autoimmunity is controversial in clinical studies as mentioned in the Introduction.

Our results clearly demonstrate that low-dose irradiation exacerbated thyroid autoimmunity in NOD-H2h4 mice as demonstrated by elevated thyroiditis scores and anti-Tg autoantibody titers. Dose, timing and area of irradiation appeared critical, that is, only 0.5 Gy whole-body irradiation one week before the beginning of NaI exhibited this enhanced effect. Irradiation restricted to the thyroid gland has no effect, indicating that this effect is systemic. Presumably irradiation to immune cells in bone marrow, spleen and/or regional lymph nodes may be critical. These data are reminiscent of the previous clinical report showing that the prevalence of autoimmune thyroid disease in atomic bomb survivors was maximum at 0.7 Sv external irradiation (e.g., whole-body irradiation) (Nagataki et al. 1994), although it is not easy to compare acute effect of irradiation on an animal model and chronic nature of irradiation effect on humans. It should also be noted here that we showed an increase in degree of thyroiditis, not incidence of thyroiditis. Since the incidence of thyroiditis is already very high (>90 %) in non-irradiated NOD-H2h4 mice, it is impossible to see whether radiation increases the incidence of thyroiditis or not in this model.

On the other hand, no irradiation effect was observed in our Graves’ model. As far as we know, there is no clinical report showing an increased incidence of Graves’
hyperthyroidism by environmental irradiation.

There are numerous basic studies on the effect of low-dose irradiation on immune response. The effect of irradiation on Th1/Th2 immune balance is contentious; some reports demonstrate increases (Shen et al. 1991), but others decreases (Bass et al. 1989, Westermann et al. 1999), in Th1/Th2 balance by irradiation. Of surprising, even the same group reported the discrepant data; Th2 polarization in a lupus model of MRL-/*lplpr mice (Tanaka et al. 2005) and in an asthma model (Fang et al. 2005), but Th1 immune deviation in the Ehrlich ascites tumor model, by repeated 0.5 Gy irradiation (Kojima et al. 2002, 2004). However, generally low-dose irradiation is reported to stimulate immune responses through various mechanisms; (i) augmentation of the proliferative response of T cells to mitogenic stimulation (Nogami et al. 1994), (ii) enhancement of primary B cell response (Anderson & Lefkovits. 1979), (iii) stimulation of autoantibody production from non-irradiated lymphocytes by irradiated cells (McGregor et al. 1979), (iv) increased macrophage function (Ibuki & Goto. 1994) and (v) increased natural killer activity and antibody-dependent cellular cytotoxicity by suppression of NOx production (Tanaka et al. 2005) and/or induction of glutathione (Kojima et al. 2004).

Nevertheless, studies with animal models of autoimmunity and tumor immunity show controversial data as mentioned in the Introduction. Differences in animal models, mouse strains, and dose and timing of irradiation in each report may be the reasons for these conflicting reports. However, again even the same irradiation protocols resulted in inconsistent data. For example, Ina et al. demonstrated that long-term low-dose-rate irradiation with 1.2 mGy/h, 23 h/day, ameliorated autoimmune disease in MRL-/*lplpr mice (Ina & Sakai. 2004) and suppressed development of
high-dose irradiation-induced thymic lymphoma (Ina et al. 2005). Similarly, Kojima’s group showed suppression of autoimmune manifestations in MRL-\textit{lpr/lpr} mice (Tanaka et al. 2005) and a delay in growth of Ehrlich ascites tumor cells by repeated 0.5 Gy irradiation (Kojima et al. 2004).

Suppression of autoimmune disease by repeated irradiation of 0.02 or 0.5 Gy was also demonstrated in MRL/MpTn-\textit{gld/gld} mice (Ootsuyama et al. 2003). These lupus-prone MRL-\textit{lpr/lpr} and MRL/MpTn-\textit{gld/gld} mice, both of which have a mutation in either Fas or Fas L gene, respectively, harbor abnormally expanded autoreactive T cells (CD3$^+$CD4$^+$CD8$^-$CD45BR/B220$^+$ and CD45BR/B220$^+$CD40$^+$) in the periphery, which are highly radiosensitive and prone to apoptosis following irradiation. This unique property may be attributed to exceptionally high-radiosensitivity of these lupus-prone mice (Tanaka et al. 2005, Ootsuyama et al. 2003).

The reasons why only low-dose irradiation given before NaI exerted its enhancing effect on autoimmune thyroiditis can not be explained by any of these previous reports or also by our FACS analysis of splenocyte subpopulations. Thus, despite the significant enhanced effect on thyroiditis, 0.5 Gy irradiation exhibited no remarkable alternations in numbers/percentages of T cell subsets (except a slight decrease in total numbers of splenocytes). Ina and Sakai demonstrated chronic low-dose-rate irradiation mediated increases in CD4$^+$ T cells and CD8$^+$ T cells and a decrease in B cells (2005). A single dose of 0.2 Gy is also reported to increase number/percentage of CD8$^+$ T cells (Hashimoto et al. 1999). In contrast, the effect of high-dose irradiation was remarkable. Thus, 3 Gy irradiation increased the ratios of Teff to Treg, an indication for enhanced immune system. Of interest, one paper shows higher radiosensitivity of Treg than Teff to high-dose (3.5 Gy) irradiation (Kipnis et al. 2004).
CD4⁺CD25⁺ Treg constitute 5~10 % of peripheral CD4⁺ T cells and has recently been demonstrated to be involved in the pathogenesis of several autoimmune diseases in humans and mice (Sakaguchi. 2005). We have also recently demonstrated importance of CD4⁺CD25⁺ Treg in the pathogenesis of thyroiditis and Graves’ hyperthyroidism in mouse models (Saitoh & Nagayama. 2006, Nagayama et al. 2007). However, the elevated ratios of Teff to Treg could not lead to enhanced immune response presumably because this irradiation dose also killed most of T cells. For any reason, again our data fits well the previous data on atomic survivors (Nagataki et al. 1994) showing the bell-shaped, convexed dose-response curve of the prevalence of autoimmune thyroid disease, not Graves’ disease, with the maximum prevalence being at 0.7 Sv.

In conclusion, we here show exacerbation of Hashimoto’s thyroiditis in a spontaneous thyroiditis model with NOD-H2ᵇᵈ mice, not of Graves’ hyperthyroidism in an induced Graves’ model with BALB/c mice, by low-dose irradiation. Although the exact mechanism(s) for this immuno-enhanced effect of low-dose irradiation for Hashimoto’s thyroiditis remain unknown, the present study is the first showing augmentation of thyroid autoimmunity by low-dose irradiation in an animal model. Further studies will be necessary to clarify the mechanism(s) for this phenomenon.
Acknowledgment

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Figure legends

**Figure 1.** Thyroiditis scores (A) and the titers of anti-Tg autoantibodies (B) in NOD-H2<sup>h4</sup> mice supplied with/without NaI in the drinking water and/or irradiated as indicated. All mice except NaI (-) group were exposed to 0.05 % NaI in the drinking water for eight weeks. Groups of mice were irradiated with different protocols (a single whole-body irradiation with 0.05, 0.5 or 3 Gy before or after the beginning of NaI, or fractionated irradiation with 0.05 Gy twice or 0.5 Gy once a week for nine weeks, starting one week before the beginning of NaI. A, thyroid glands were fixed, embedded and sections stained with hematoxylin-eosin to score thyroiditis (see the Methods). B, the titers of anti-Tg antibodies were determined by ELISA as described in the Materials and methods. Data are mean ± S.D. *, p < 0.05 compared to controls. **, p <0.01 compared to controls.

**Figure 2.** Representative histology of the thyroid glands in NOD-H2<sup>h4</sup> mice. A, grade 0; B, grade 2; C, grade 4. Magnification, x100.

**Figure 3.** Thyroiditis scores in NOD-H2<sup>h4</sup> mice supplied with NaI in the drinking water and/or irradiated as indicated. All the mice were exposed to 0.05 % NaI in the drinking water for eight weeks. Groups of mice were first irradiated with 0.05 Gy once one or two weeks before a single whole-body irradiation with 0.5 Gy. In another group of mice, only the thyroid glands were irradiated with 0.5 Gy. Thyroid glands were fixed, embedded and sections stained with hematoxylin-eosin to score thyroiditis (see the Methods). Data are mean ± S.D. *, p < 0.05 compared to controls. **, p
<0.01 compared to controls.

**Figure 4.** Serum T₄ concentrations (A) and the titers of anti-TSHR antibodies (B) in untreated BALB/c mice and those immunized with AdTSHR289 and/or irradiated as indicated. Mice were untreated or immunized with AdTSHR289. Groups of mice were irradiated with different protocols (a single whole-body irradiation with 0.05 or 0.5 Gy before or after the immunization, or fractionated irradiation with 0.05 Gy, twice a week for five weeks, starting one week before the immunization). T₄ and antibody levels were determined by RIA and flow cytometry, respectively, as described in the Materials and methods. A horizontal line depicts normal upper limits of T₄ values. Data are shown for individual mice (A) or as mean ± S.D. (B).

**Figure 5.** Representative histology of the thyroid glands in BALB/c mice. A, control euthyroid; B, immunized hyperthyroid. Magnification, x100.

**Figure 6.** Flow cytometric analysis of CD4, CD8, CD19, CD25 and Foxp3 expression on splenocytes of control and irradiated NOD-H₂hra mice. Three to four days after irradiation, splenocytes were analyzed. Representative data obtained from control mice and those irradiated with 3 Gy are shown.

**Figure 7.** Spleen weights, number of splenocytes, percentages of CD4⁺, CD8⁺, CD4⁺CD25⁺ and CD4⁺Foxp3⁺ T cells and CD19⁺ B cells, and the ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells and CD4⁺Foxp3⁺ T cells to CD4⁺ T cells following irradiation. Spleen from untreated mice and those irradiated with a single irradiation of 0.05, 0.5 or
3 Gy three to four days before were weighted. Splenocytes were stained with various cell surface markers for T cell subpopulations and analyzed by flow cytometry. Note that the similar results were obtained when spleen was obtained seven days after irradiation. Data are means ± S.D. (n = 3–6). *, p < 0.05 compared to controls. **, p < 0.01 compared to controls.
References


of childhood disease in Belarus associated with the Chernobyl accident. Environmental Health Perspectives 105(Suppl. 6): 1529-1532.


Saitoh O, Nagayama Y. 2006. Regulation of Graves’ hyperthyroidism with naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells in a mouse model. Endocrinology 147: 2417-2422.


Figure 1

Nagayama Y, et al. Figure 1
Nagayama Y, *et al.* Figure 2
Nagayama Y, et al. Figure 3

Figure 3: Thyroiditis scores

- No radiation
- 0.5 Gy x1 before NaI
- 0.05 Gy 1 wk prior to 0.5 Gy x1 before NaI
- 0.05 Gy 2 wks prior to 0.5 Gy x1 before NaI
- 0.5 Gy x1 before NaI (thyroid)

Thyroiditis scores range from 0 to 4. The number of samples per condition is indicated in parentheses: (n=8), (n=8), (n=8), and (n=9). The graph shows statistically significant differences between the groups, indicated by * and ** symbols.
Free T₄ (ng/dl)

- untreated
- No treatment
- 0.5 Gy x1 before
- 0.5 Gy x1 after
- 0.05 Gy x1 before
- 0.05 Gy x1 after
- 0.05 Gy x12

Ad-TSHR289

Anti-TSHR Ab titer (MFI)

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Nagayama Y, et al. Figure 6

CD8+ T cells

CD19+ B cells

CD4+CD25+

CD4+Foxp3+

0 3 (Gy)
Nagayama Y, et al. Figure 7

- **spleen weights (mg)**
  - 100
  - 50
  - 25
  - 10
  - 5
  - 2
  - 1

- **spleenocytes (x10^7)**
  - 10
  - 5
  - 2.5
  - 1
  - 0.5
  - 0

- **CD4\(^+\) T cells (%)**
  - 20
  - 10
  - 5
  - 2
  - 1

- **CD4\(^+\)CD25\(^+\)/CD4\(^+\) (%)**
  - 30
  - 20
  - 10
  - 5
  - 2
  - 1

- **CD4\(^+\)Foxp3\(^+\) (%)**
  - 10
  - 5
  - 2
  - 1

- **CD8\(^+\) T cells (%)**
  - 20
  - 10
  - 5
  - 2
  - 1

- **CD4\(^+\)CD25\(^+\) T cells (%)**
  - 10
  - 5
  - 2
  - 1

- **CD4\(^+\)Foxp3\(^+\)/CD4\(^+\) (%)**
  - N.D.
  - N.D.
  - N.D.
  - N.D.

- **CD19\(^+\) B cells (%)**
  - N.D.
  - N.D.
  - N.D.
  - N.D.

- **CD8\(^+\) T cells (%)**
  - N.D.
  - N.D.
  - N.D.
  - N.D.

- **CD4\(^+\)CD25\(^+\) T cells (%)**
  - N.D.
  - N.D.
  - N.D.
  - N.D.

- **CD4\(^+\)Foxp3\(^+\)/CD4\(^+\) (%)**
  - N.D.
  - N.D.
  - N.D.
  - N.D.