Original Article

Mechanisms of action of radon therapy on cytokine levels in normal mice and rheumatoid arthritis mouse model

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The typical indication of radon therapy is rheumatoid arthritis. Although there are several reports that radon therapy has regulation effects on Th17 cells, there has been no study reporting that radon inhalation affects the immune balance among Th1, Th2, and Th17. The purpose of this study is to examine the cytokine changes after radon inhalation. BALB/c mice inhaled radon at 2,000 Bq/m³ for 2 or 4 weeks. SKG/Jcl mice inhaled radon at 2,000 Bq/m³ for 4 weeks after zymosan administration. The results showed that radon inhalation for 4 weeks activated the immune response of Th1, Th2, and Th17. Moreover, the balance among them was not lost by radon inhalation. Radon inhalation for 4 weeks decreased superoxide dismutase activity and increased catalase activity in spleen. These findings suggest that an imbalance of oxidative stress may contribute to activate the immune response. Although zymosan administration activated Th17 immune response and decreased Th1 and Th2 immune response in SKG/Jcl mice, most cytokines related to Th1, Th2, and Th17 approached the normal level by radon inhalation. These findings suggested that radon inhalation has a different action between SKG/Jcl mice and normal BABL/c mice. This may indicate that radon inhalation has an immunomodulation function.

***Key Words*: radon, cytokine, oxidative stress, rheumatoid arthritis, immunomodulation function**

Radon therapy is a traditional therapy. Clinical studies revealed that radon therapy alleviated pain related diseases, such as rheumatoid arthritis (RA).[1,2] In recent years, Maier reviewed the mechanisms of the effects of radon therapy.[3] The initial action of the proposed mechanisms by radon inhalation is to increase antioxidant substances such as superoxide dismutase (SOD),[4,5] which leads to a decrease in reactive oxygen species (ROS). For example, peaks of SOD activity were observed under specific conditions in various studies.[4,5] Therefore, activation of antioxidative functions induced by radon inhalation depends on the radon concentration or time after radon inhalation.

A further effect of radon therapy is to inhibit inflammation. ROS play an important role in the development of inflammation. For example, carrageenan or formalin administration to the mouse paw induced inflammatory edema or inflammatory pain due to the production of ROS.[6,7] We have reported that radon inhalation inhibits inflammation induced by carrageenan[8] and formalin[9] administration to mice paw via the activation of antioxidative functions. Similarly, there is a lot of evidence that oxidative stress is closely related to RA.[10,11] In clinical studies, radon therapy with RA patients reduces pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, suggesting the relief of inflammation.[12] The probable mechanism is that radon therapy increases Treg cells, which regulate Th17 cells.[13] Consequently, the receptor activator of nuclear factor-kappa B ligands, which leads to the activation of osteoclast, decreases after radon therapy.[12] Thus, the activation of antioxidative functions following radon inhalation may contribute to the alleviation of inflammation.

Although there are several reports that radon therapy has regulation effects on Th17 cells,[13] there has been no study reporting that low-dose radon inhalation affects the immune balance among Th1, Th2, and Th17. Clarifying the changes in cytokines by radon inhalation might help understand the mechanisms of radon therapy. The purpose of the present study is to examine the cytokine changes by radon inhalation and the difference between normal BALB/ mice and SKG/Jcl mice, which are RA model mice derived from BALB/ mice.

Materials and methods

Animals. Eight-week-old male BALB/c mice and 10-week-old SKG/Jcl mice (CLEA Japan Inc., Tokyo, Japan) were housed under room temperature and a preset light-dark cycle of 12:12 h. Ethics approval was obtained from the Animal Care and Use Committee of Okayama University.

Zymosan administration. Two mg of zymosan was dissolved in 500 ml of saline solution. Mice were subjected to intraperitoneal injection of zymosan before radon inhalation.

Radon inhalation. BALB/c mice inhaled radon at a concentration of 2,000 Bq/m^3 for 2 or 4 weeks. After zymosan administration, SKG/Jcl mice inhaled radon at a concentration of 2,000 Bq/m^3 for 4 weeks using our developed radon inhalation system for small animals. The control group received sham inhalation only.

Sample collection. The spleens of BALB/c mice and lungs of SKG/Jcl were removed quickly after euthanasia using CO_2. The blood was collected from the heart and centrifuged at 3,000 × *g* for 5 min at 4°C. The upper aqueous layers were collected for the assay of cytokines.

Cytokine assay. The level of cytokines, such as interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-17A, interferon (IFN)-γ, the granulocyte macrophage colony-

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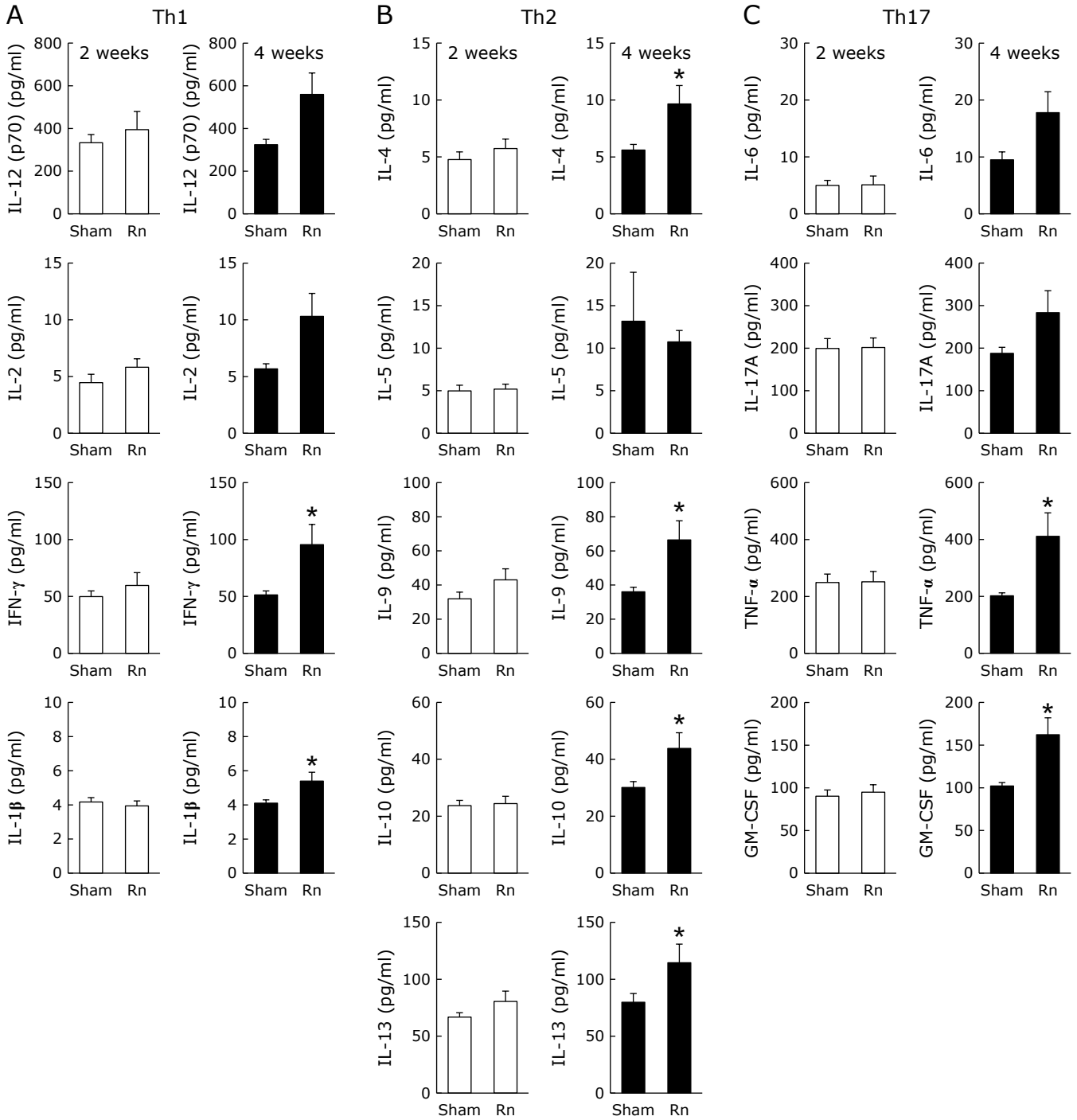

Fig. 1. Changes in (A) Th1-, (B) Th2-, and (C) Th17-related cytokines in serum of BALB/c mice. The number of each experimental group is 7. Data are presented as mean ± SEM. *p<0.05 vs Sham.

stimulating factor (GM-CSF), and TNF-α, was determined using an assay kit (Bio-Plex Pro, Bio-rad, CA). The assay was consigned to Okayama University Hospital Biobank.

Biochemical assays. For SOD, catalase (CAT), and total glutathione (t-GSH) assays, samples spleens (BALB/c mice) and lungs (SKG/Jcl mice) were homogenized in 10 mM phosphate-buffered saline (PBS; pH 7.4), and homogenates were used for analyses. The activity of SOD and CAT and t-GSH contents were measured following the method described in our previous studies.[14,15]

Assessment of Arthritis Score. The Arthritis Score was determined based on the method by Hata *et al.*[16] The scores showed the following; 0: no joint swelling, 0.1: swelling of one finger joint, 0.5: mild swelling of the wrist or ankle, and 1.0: severe swellings of the wrist or ankle.

Statistical analyses. The data values are presented as the mean ± SEM. The statistical significance of differences was determined by an unpaired t test for comparison between two groups or one-way repeated-measures analysis of variance (ANOVA) and Tukey's test for multiple comparisons, where appropriate. P values <0.05 were considered significant.

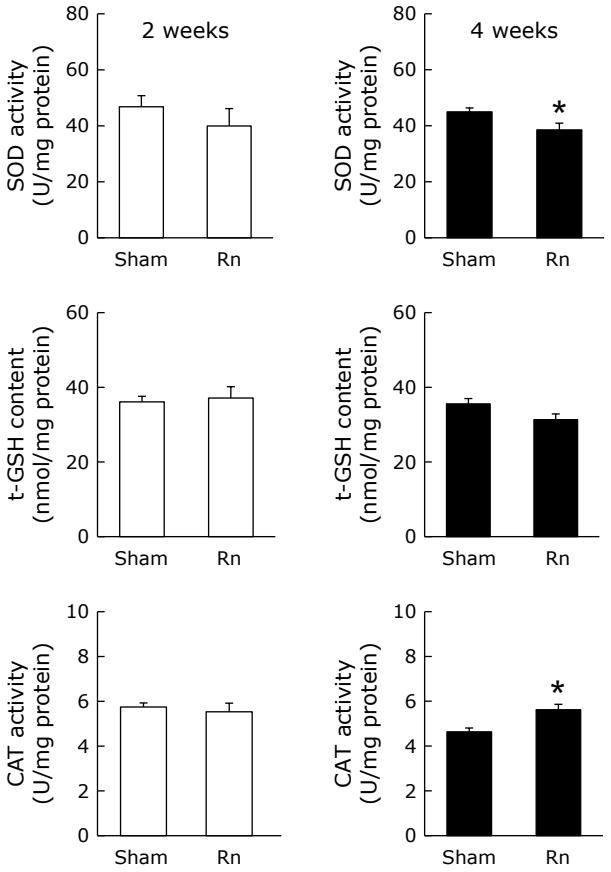

Fig. 2. Changes in antioxidative functions in spleen of BALB/c mice. The number of each experimental group is 7. Data are presented as mean ± SEM. *p<0.05 vs Sham.

Results

Changes in Th1 related cytokines in serum of BALB/c mice. Radon inhalation at a concentration of 2,000 Bq/m^3 for 2 weeks did not change the levels of IL-12(p70), IL-2, IFN-γ, and IL-1β. However, radon inhalation significantly increased the levels of IFN-γ and IL-1β after 4 weeks of radon inhalation (Fig. 1A).

Changes in Th2 related cytokines in serum of BALB/c mice. Radon inhalation at a concentration of 2,000 Bq/m^3 for 2 weeks did not change the levels of IL-4, IL-5, IL-9, IL-10, and IL-13. However, radon inhalation significantly increased the levels of IL-4, IL-9, IL-10, and IL-13 after 4 weeks of radon inhalation (Fig. 1B).

Changes in Th17 related cytokines in serum of BALB/c mice. Radon inhalation at a concentration of 2,000 Bq/m^3 for 2 weeks did not change the levels of IL-6, IL-17A, TNF-α, and GM-CSF. However, radon inhalation significantly increased the levels of TNF-α and GM-CSF after 4 weeks of radon inhalation (Fig. 1C).

Changes in antioxidative substances in spleen of BALB/c mice. Radon inhalation at a concentration of 2,000 Bq/m^3 for 2 weeks did not change the levels of SOD, CAT, and t-GSH in spleen. However, radon inhalation significantly decreased SOD activity and significantly increased CAT activity after 4 weeks of radon inhalation (Fig. 2).

Changes in Th1, Th2, and Th17 related cytokines in serum of SKG/Jcl mice. Zymosan administration significantly decreased IL-12(p70), which was related to Th1 differentiation, and IL-4, which was related to Th2 differentiation. In contrast, IL-6, which was related to Th17 differentiation, increased by zymosan administration. Radon inhalation at a concentration of 2,000 Bq/m^3 after zymosan administration significantly decreased IL-9 levels. Sham or radon inhalation after zymosan administration significantly decreased the levels of TNF-α and GM-CSF (Fig. 3).

Changes in antioxidative substances in lungs of SKG/Jcl mice. No significant changes were observed in the activities of SOD and CAT and t-GSH contents in lungs (Fig. 4).

Arthritis score of SKG/Jcl mice. On day 21, the arthritis scores of zymosan-administrated mice without radon inhalation were significantly higher than those of control mice. On day 28, the arthritis scores of zymosan-administrated mice with/without radon inhalation were significantly higher than those of control mice (Fig. 5).

Discussion

IL-12 is an inducer of Th1 immune response, which leads to cell-mediated immunity. Moreover, the activation of Th1 immune response results in the production of natural killer T (NKT) cells by cytokines, such as IL-2 and IFN-γ.[17] A report showed that low-dose γ-irradiation enhances NKT activity via the increase of reduced glutathione.[18] Another report suggests that radon hot spring drinking modulates the immunity balance by activating cell-mediated immunity in DNP-Ascaris-immunized mice.[19] In the present study, radon inhalation for 4 weeks increased the Th1-related cytokines, suggesting this inhalation increased the Th1 immune response. Although we did not assay NKT activity, it could be speculated that the effects of radon inhalation were similar to those of γ-irradiation.

IL-4 is an important cytokine to promote Th2 cell differentiation. Moreover, Th2 induces humoral immune response and is closely related to the production of immunoglobulin E (IgE).[20] A report suggests that oxidative stress promotes differentiation toward Th2.[21] We have reported that low-dose (0.25 Gy) X-irradiation increased plasma cells in the spleen of mice, while high-dose (15 Gy) X-irradiation decreased plasma cells.[22] These findings indicate that Th2 immune responses depend on the radiation doses. Since it is well known that radiation is also a source of oxidative stress, radon inhalation probably affects the Th2 immune response. Our results showed that radon inhalation for 4 weeks significantly increased Th2-related cytokines, suggesting that this inhalation activated the Th2 immune response. However, radon inhalation for 2 weeks did not increase these cytokines. These findings indicate that radon inhalation for 4 weeks may activate the Th2 immune response, similar to low-dose X-irradiation.

Transforming growth factor-beta (TGF-β) and IL-6 play an important role in Th17 cell differentiation.[23–25] In the present study, radon inhalation increased the IL-6 level, suggesting the promotion of the Th17 immune response. In fact, the levels of Th17-related cytokines increased by radon inhalation. Taken together, radon inhalation activated the immune response of Th1, Th2, and Th17. Moreover, the balance among them was not lost by radon inhalation. These are important findings of the present study.

Mitogen-activated protein kinase 1 works as an oxidative-stress sensor, which results in the production of IL-6 under oxidative stress conditions.[26] Another report suggests that ROS induces cytokines, such as IL-6, TNF-α, and macrophage inflammatory protein-2.[27] On the other hand, we have reported that radon inhalation activates antioxidative functions in mouse organs.[5] For example, radon inhalation at 2,000 Bq/m^3 for 1 day inhibited carbon tetrachloride-induced hepatopthy via activation of antioxidative functions.[28] Similarly, radon inhalation at 2,000 Bq/m^3 for 7 days inhibited colitis in mice induced by dextran sulfate sodium administration.[29] Thus, radon inhalation can

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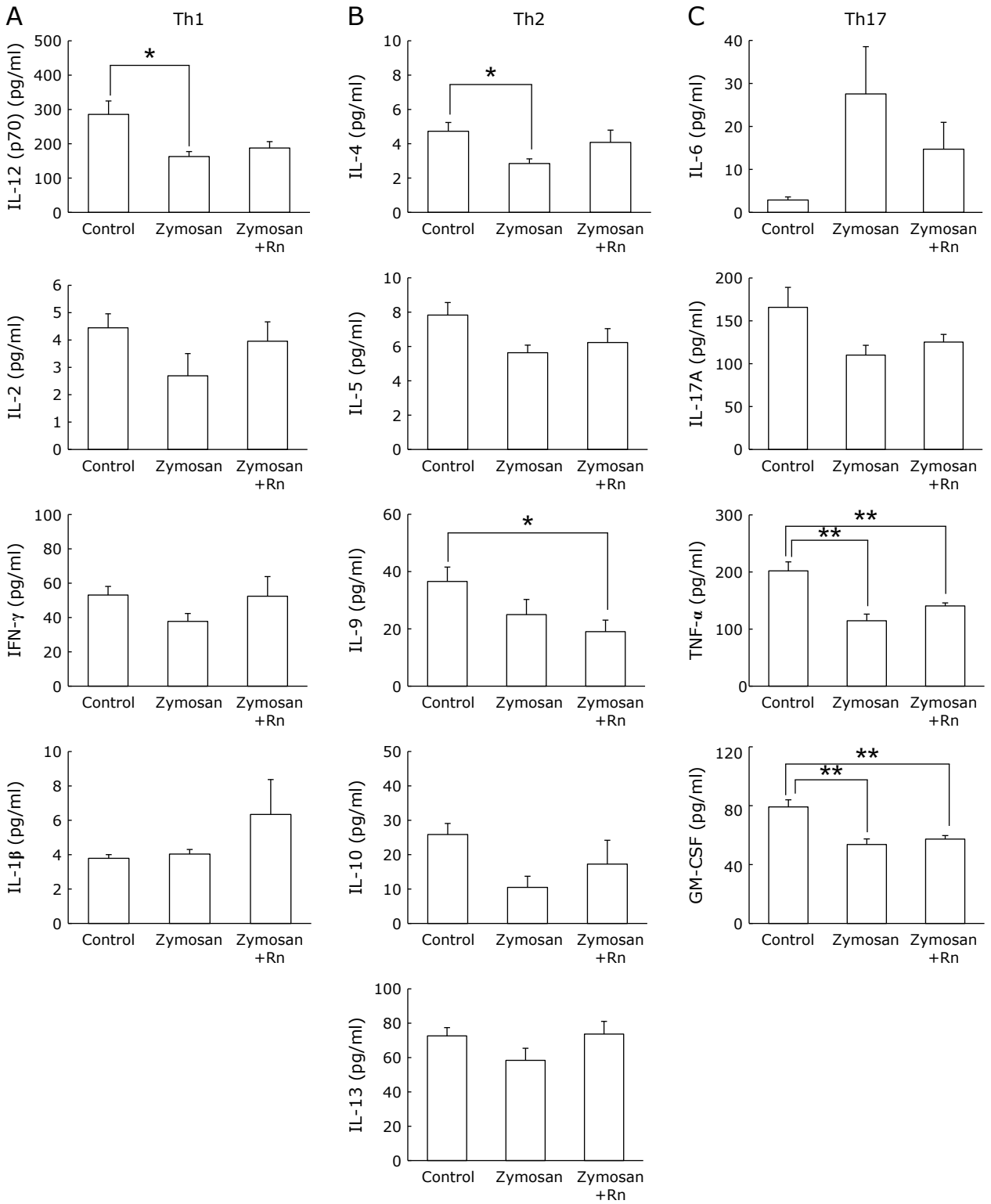

Fig. 3. Changes in (A) Th1-, (B) Th2-, and (C) Th17-related cytokines in serum of SKJ/Jcl mice. The number of each experimental group is 6–7. Data are presented as mean ± SEM. *p<0.05, **p<0.01.

inhibit ROS-induced damages, such as inflammation. Therefore, the evaluation of antioxidative functions may give a hint why radon inhalation increased cytokines. While radon inhalation for 2 weeks did not change the activities of SOD and CAT and t-GSH contents, radon inhalation for 4 weeks significantly decreased SOD activity and significantly increased CAT activity. These findings might suggest that active oxygen is disproportionate and detoxified.

The development of RA is closely related to the Th17 immune response.[30] Zymosan administration significantly decreased IL-12(p70), which was related to Th1 differentiation, and IL-4, which was related to Th2 differentiation. In contrast, this

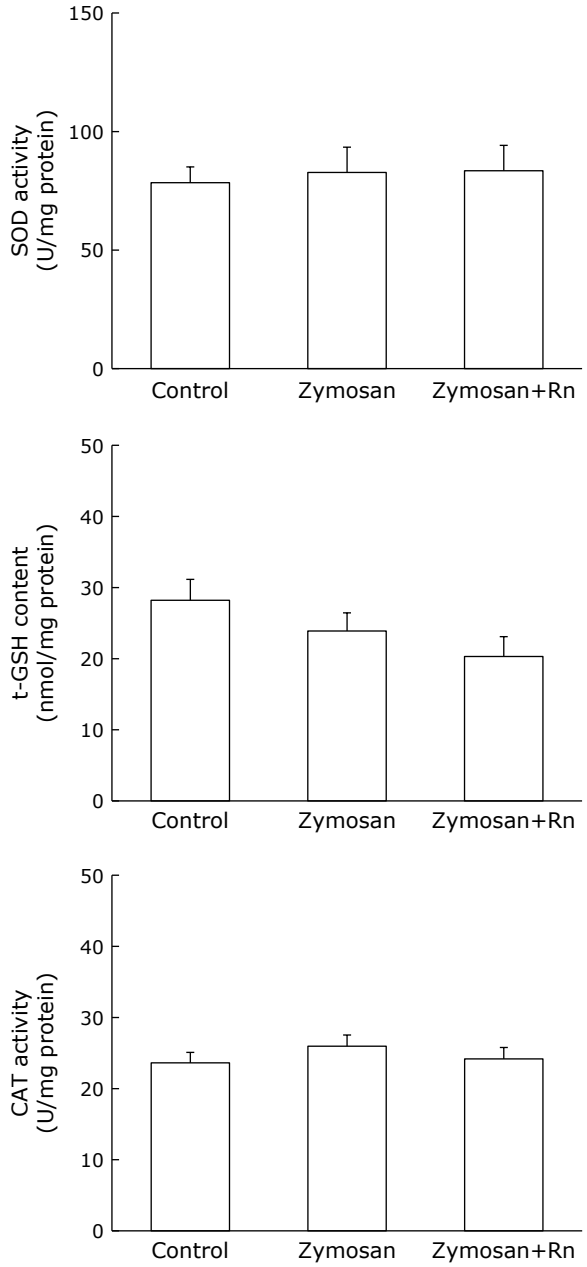

Fig. 4. Changes in antioxidative functions in the lungs of SKG/Jcl mice. The number of each experimental group is 6–7. Data are presented as mean ± SEM.

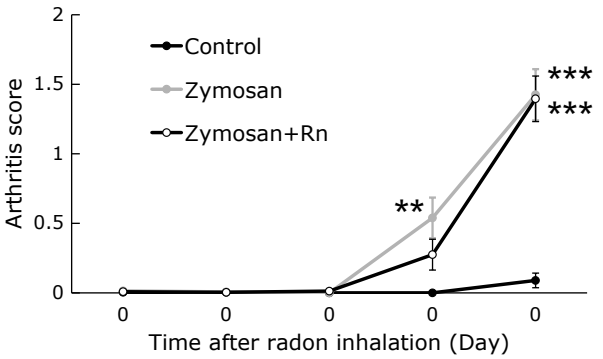

Fig. 5. Arthritis score of SKG/Jcl mice. The number of each experimental group is 6–7. Data are presented as mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$ vs control.

increased IL-6, which was related to Th17 differentiation, and this suggested that zymosan administration resulted in a loss of the balance among Th1, Th2, and Th17 and promoted the Th17 immune response, which leads to RA. On the other hand, the levels of most cytokines approached the control level after radon inhalation. Interestingly, the phenomenon was completely different for normal mice. This may suggest that radon inhalation has an immunomodulatory effect, and this may be the mechanism by which effects of RA are alleviated, which is an autoimmune disease. Although the arthritis scores of zymosan-administrated mice with/without radon inhalation on day 28 were almost the same, the scores of mice that inhaled radon was lower than that of zymosan-administrated mice on day 21. This finding suggested that radon inhalation for 3 weeks was much more effective in inhibiting RA.

ROS play an important role in developing RA.[31] In fact, gelatin-conjugated SOD, which is a scavenger of superoxide anion, suppresses collagen-induced arthritis.[32] A report suggested that RA is associated with the development of oxidative stress in lungs.[33] Although there were no significant differences in antioxidative functions in lungs among each group in the present study, radon therapy clearly alleviates RA symptoms.[1,2] Therefore, optimization of radon inhalation conditions may be needed.

In this regard, we previously reviewed the enhancement of antioxidant functions by low-dose radiation and its applicability to the treatment of ROS-related diseases.[34] We further reviewed recent research for the efficacy, mechanism, and new indications of radon therapy.[35] Moreover, we reported that the DNA damage in mouse organs due to excess ROS was suppressed by radon inhalation.[36] On the other hand, it has been suggested that the amount of melanin-derived radicals in the skin may be an endogenous marker for the health effects of long-term low-dose radiation.[37] In the future, it may be necessary to consider this method to confirm the safety of the long-term use of radon therapy.

In conclusion, radon inhalation activates the immune response of Th1, Th2, and Th17 in normal BALB/c mice probably due to an imbalance of the redox state. In contrast, radon inhalation has immunomodulation effects in SKG/Jcl mice, which are RA model mice. In this way, the action of radon inhalation between SKG/Jcl mice and BABL/c mice was different. This may indicate that radon inhalation has an immunomodulation function because RA is an autoimmune disease. Further studies are needed to clarify the alleviation mechanisms for RA by radon inhalation. It is also important to consider the relationship between the RA remedy agent used as a treatment for COVID-19 and the typical indication for radon therapy, i.e., RA.

Author Contributions

TK: study design, biochemical assay, drafting of the manuscript. SN, KM, RY, YF: biochemical assay. AS: measurement of radon concentration. NK, FM: study design. KY: study design and supervision.

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Abbreviations

CAT	catalase
GM-CSF	granulocyte macrophage colony-stimulating factor
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
NKT	natural killer T
RA	rheumatoid arthritis
ROS	reactive oxygen species
SOD	superoxide dismutase
t-GSH	total glutathione
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor α

Conflict of Interest

No potential conflicts of interest were disclosed.

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