

Foxp3⁺-Treg Cells Enhanced by Repeated Low-Dose Gamma-Irradiation Attenuate Ovalbumin-Induced Allergic Asthma in Mice

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Gamma radiation is used for several therapeutic indications such as cancers and autoimmune diseases. Low-dose whole-body γ irradiation has been shown to activate immune responses in several ways, however, the effect and mechanism of irradiation on allergic asthma remains poorly understood. This study investigated whether or not irradiation exacerbates allergic asthma responses and its potential mechanism. C57BL/6 mice were sensitized and challenged with ovalbumin (OVA) to induce asthma. The mice received whole-body irradiation once daily for 3 consecutive days with a dose of 0.667 Gy using ¹³⁷Cs γ rays 24 h before every OVA challenge. Repeated low-dose irradiation reduced OVA-specific IgE levels, the number of inflammatory cells including mast cells, goblet cell hyperplasia, collagen deposition, airway hyperresponsiveness, expression of inflammatory cytokines, CCL2/CCR2, as well as nuclear factor kappa B (NF- κ B) and activator protein-1 activities. All of these factors were increased in BAL cells and lung tissue of OVA-challenged mice. Irradiation increased the number of Treg cells, expression of interleukin (IL)-10, IL-2 and IL-35 in BAL cells and lung tissue. Irradiation also increased Treg cell-expressed Foxp3 and IL-10 by NF- κ B and RUNX1 in OVA-challenged mice. Furthermore, while Treg cell-expressing OX40 and IL-10 were enhanced in lung tissue or act-bone marrow-derived mast cells (BMMCs) with Treg cells, but BMMCs-expressing OX40L and TGF- β were decreased. The data suggest that irradiation enhances Foxp3⁺- and IL-10-producing Treg cells, which reduce OVA-induced allergic airway inflammation and tissue remodeling through the down-regulation of migration by the CCL2/CCR2 axis and activation of mast cells via OX40/OX40L in

lung tissue of OVA-challenged mice. © 2013 by Radiation Research Society

INTRODUCTION

Radiation hormesis has been observed at low doses of ionizing radiation and low-dose irradiation has been used for investigations in physiology, immunology, medical diagnosis and therapy (1).

Depending on the dose and target cell type, γ irradiation can induce apoptosis, encourage survival or promote differentiation of immune cells (2). Low-dose whole-body irradiation appears to activate immune responses in several ways, leading in some cases to faster wound healing and increased resistance to toxins, infections and tumor cell injection (1, 3). Low-dose irradiation has been shown to attenuate some chronic diseases including low-grade non-Hodgkin's lymphoma (4), gingivitis (5), malignant cancers and autoimmune symptoms in MRL-lpr/lpr mice (6). Irradiation has also been shown to attenuate experimental autoimmune encephalomyelitis (7) and collagen-induced arthritis without direct damage to lymphocytes (8) by the up-regulation of Treg cells (9). In addition, ovalbumin (OVA) that has been modified by γ irradiation appears to suppress humoral and cellular immune responses (10). However, exacerbation of asthma by repeated low-dose irradiation has been previously described (11). Furthermore, low-dose irradiation has been shown to stimulate tumor necrosis factor- α (TNF- α)-induced ICAM-1 mRNA expression in human coronary vascular cells (12), to alter the changes of the cytokine milieu (13), modulate matrix metalloproteinase-9 production in macrophage cell line (14), and inhibit the release of mediators by the reduction of Fc ϵ RI expression in mast cells (15).

Allergic asthma is a chronic and complex inflammatory disease of the airways that is characterized by reversible obstruction of airway hyperresponsiveness (AHR), infiltration of inflammatory cells into lung tissues, mucus overproduction (16), and the overexpression of Th2-mediated cytokines and chemokines (17). The release of

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Th2-mediated cytokines causes persistent inflammatory cell recruitment and induces structural changes in airway walls such as increased basement membrane thickness, increased collagen deposition, smooth muscle hypertrophy and goblet cell hyperplasia (18).

Mast cells play an important role in various inflammatory disorders such as allergic and chronic immune diseases. Circulating mast cell progenitors (MCp) are released from bone marrow, accumulate in different tissues, mature under the influence of locally produced factors, and are recruited to the inflammatory sites through expression of the CCL2/CCR2 axis (19). Interleukin (IL)-10-induced lung fibrocyte recruitment also is CCL2/CCR2 specific (20).

Treg cells, which function mainly to inhibit immune responses and protect against the development of chronic immune diseases, appear to be necessary to maintain peripheral tolerance to self-antigens. Recently, it was reported that Treg cells block the influx of effector T cells into inflamed tissues in a cytokine-dependent manner (21), and suppress the production of allergen-specific IgE, and induce IgG4 (22). This suppression has the potential to control the development of allergic diseases by altering the ongoing sensitization and effector phase by several major pathways such as the suppression of effector Th1, Th2 and Th17 cells (23–26). Treg also suppresses allergic inflammation through direct action on mast cells, basophils and eosinophils, and tissue remodeling via IL-6 and/or OX40/OX40L (27, 28). Treg cell transcription factor Foxp3 expression is regulated by multiple transcription factors such as nuclear factor of activated T-cells (NFAT), activator protein-1 (AP-1), STAT5, cAMP response element-binding protein (CREB) and nuclear factor-kappa B (NF- κ B), all of which modulate Treg cell development (26, 29, 30), and RUNXs, which control the induction and suppressive function of Treg cells (25, 29). Thus, strategies to induce allergen-specific Treg activity hold great promise for the treatment and prevention of asthma.

Based on the aforementioned reports that low-dose irradiation attenuates various chronic diseases by Treg cell up-regulation and that Treg cells plays an important role in immune regulation of allergic diseases, we hypothesized that irradiation could enhance Treg cells, and that the enhanced Treg cells may reduce OVA-specific allergic asthma symptoms in mice.

We observed that irradiation itself and/or Treg cells induced to migrate by irradiation reduced allergic airway inflammation and tissue remodeling through down-regulating the migration of inflammatory cells, including mast cells, via cytokines/chemokines and through inactivation of mast cells by OX40/OX40L interaction.

MATERIALS AND METHODS

Animals

Female, 8-week-old C57BL/6 mice weighing 20 g were obtained from Orient Bio Inc. (Seongnam, Korea) and maintained in specific

pathogen-free conditions. All animals were housed in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care. Additionally, all protocols were approved by the Institutional Review Board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University.

Allergy Induction with OVA

The experimental mice were divided into four groups ($n = 8$ mice per group). A negative control group of mice (Con) were sensitized and nebulized with phosphate buffered saline (PBS). In the second group (irradiation alone), mice were sensitized and nebulized with PBS, and irradiated three times at a dose of 0.667 Gy (total dose, 2 Gy). In the third group (OVA), mice were sensitized with OVA. In the fourth group (OVA + 2 Gy), mice were sensitized and nebulized with OVA prior to irradiation, described above.

OVA sensitization was performed with 20 μ g OVA (Grade V; Sigma-Aldrich, St. Louis, MO) conjugated with 50 μ g/200 μ l aluminum (aluminum hydroxide, 2% Alhydrogel; Superfos Biosector, Vedbaek, Denmark) by intraperitoneal (i.p.) injection on days 1 and 15. After 5 days, mice were nebulized with 5% OVA in PBS for 30 min once daily for 3 consecutive days, from days 20 to 22 (OVA challenge), using a nebulizer (Mega Medical, Seoul, Korea). The control mice were sensitized and nebulized with PBS at the same time. All mice were sacrificed on day 23 (18).

Exposure of γ -Ray Irradiation

Mice were placed in an Aperture Lucite Cabinet (CIS Biointernational, Saclay, France) and received whole-body irradiation without anesthesia once daily for 3 consecutive days (days 19 to 21 for irradiation) at a individual dose of 0.667 Gy (2 Gy total dose; dose rate 4.72 Gy/min) using a ^{137}Cs γ ray 24 h before every OVA challenge (days 20–22 for OVA challenge) (Supplementary Fig. 1A; <http://dx.doi.org/10.1667/RR3082.1.S1>).

Bronchoalveolar Lavage (BAL) Fluid

BAL fluid was collected, stained with Diff-Quick (International Reagents, Kove, Japan) and counted by microscopy (18). Mast cells were stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany) and quantified at five randomly selected 200 $\times$ 200 μ m areas (5 sites per slide $\times$ 8 mice = 40 sites) by microscopy (31) (Supplementary Information; <http://dx.doi.org/10.1667/RR3082.1.S3>).

Lung Tissue Histology

Lung tissue sections (3 μ m) were stained with hematoxylin and eosin (H&E) for general morphology, with periodic acid Schiff (PAS) for goblet cells (18), and with van Gieson (Sigma-Aldrich) to visualize collagen deposition (32). The amount of collagen was determined by a Sircol collagen assay (Supplementary Information; <http://dx.doi.org/10.1667/RR3082.1.S3>) (32).

Measurement of OVA-Specific Serum IgE

Blood samples were collected immediately after anesthesia by cardiac puncture. Blood was allowed to clot at room temperature and was then centrifuged at 550g for 30 min. Aliquots of serum were stored at -70°C until OVA-specific serum IgE analysis using ELISA (Alpha Diagnostic International, San Antonio, TX) (18). Briefly, for OVA-specific IgE analysis, microplates were coated with purified rat anti-mouse IgE and were then treated with mouse sera followed by biotinylated OVA prepared with a peroxidase-conjugated goat anti-biotin secondary antibody. Reactions were read using an ELISA plate reader at 450 nm. Mouse IgE was used for calibrating the assay.

Measurement of AHR

AHR was assessed by measuring changes in the airway resistance (R_L , cmH₂O/ml/s) after mice were treated with methacholine (MCh, Sigma-Aldrich) using the Flexivent system (SCIREQ, Montreal, PQ, Canada). Anesthetized (pentobarbital sodium 70–90 mg/kg, i.p. injection) and tracheostomized (18G cannula) mice were mechanically ventilated (160 breaths/min, tidal volume of 10 ml/kg, positive end expiratory pressure of 3 cmH₂O). Increasing concentrations (3–25 mg/ml) of MCh aerosol were administered by nebulization (3–25 mg/ml). Airway resistance was measured at 60 and 120 s, respectively, after the administration of MCh (18). Provocative concentration (PC_{300}) was calculated by the dose of MCh that induced a 300% increase in R_L .

Immunoblotting Analysis

Then (1×10^6 cells), lung tissues (50 mg/50 μ l), Treg cells (4×10^5 cells), or bone marrow-derived mast cells (BMMCs, 1×10^6 cells) were suspended in a low-salt lysis buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride (PMSF), 2.0 μ g/ml aprotinin, and 2.0 μ g/ml leupeptin, and were allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron apparatus (Kinematica, Littau, Switzerland). After centrifugation, supernatants (20 μ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis and the resolved proteins were electrophoretically transferred to nitrocellulose membranes (Amersham BioSciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1% Tween 20 (PBST) and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with antibodies against γ -H2AX, EMBP, Muc5ac, Foxp3, RUNX, CCL2, CCR2, OX40 or OX40L diluted with PBST to 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with PBST and treated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG diluted to 1:5,000–1:10,000 (BD Pharmingen, Franklin Lakes, NJ) in PBST for 60 min. After washing, the protein bands were visualized by enhanced chemiluminescence using a chemiluminometer (Fuji Film, Tokyo, Japan) (18).

RT-PCR

Total cellular RNA was isolated from BAL cells (1×10^6 cells), lung tissues (50 mg/50 μ l), Treg cells (4×10^5 cells) or BMMCs (1×10^6 cells) using Trizol reagent (Molecular Research Center, Cincinnati, OH). RT-PCR was performed in a final volume of 50 μ l using an amfiRivert 1-Step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (Bioer Technology, Hangzhou, China). PCR was performed for 35 cycles. The primer sequences used are provided in Supplementary Information (<http://dx.doi.org/10.1667/RR3082.1.S3>) (18).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from BAL cells (1×10^6 cells), lung tissues (50 mg/50 μ l) or Treg cells (4×10^5 cells), and EMSA was performed using a previously described method (Supplementary Information; <http://dx.doi.org/10.1667/RR3082.1.S3>) (18).

Isolation and Activation of T Cells

T cells were isolated from lung tissues and spleens of OVA-challenged mice (*in vivo*) or from spleens of normal mice (*in vitro*) using Dynal® Mouse T Cell Negative Isolation Kit (Invitrogen, Carlsbad, CA), and confirmed by one-color flow cytometric analysis using anti-CD3-PE mAb (eBioscience, San Diego, CA) (33). Purity for total T cells was more than 92%.

Treg cells and act-T cells included in total T cells from lung tissues or spleens of OVA-challenged mice were analyzed using Mouse Depletion Dynabeads®. Round-bottomed 96-well plates coated with 3 μ g/ml anti-CD3 in PBS overnight at 4°C were cultured with 5×10^4 T cells isolated from normal splenocytes for 2 h at 37°C. The plates received irradiation once daily for 3 consecutive days at a dose of 0.667 Gy (total 2 Gy; dose rate, 4.73 Gy/min). The irradiated cells were stimulated with 5 μ g/ml anti-CD28. After 72 h, the harvested cells were analyzed using a FACSCalibur flow cytometer and CellQuest Software (Becton Dickinson, San Jose, CA) (34).

Flow Cytometry Isolation of Treg Cells

Treg cells were separated using manufacturer's instructions (Invitrogen). Briefly, BAL cells (1×10^6 cells) or splenocytes (1×10^8 cells) were resuspended in 1 ml isolation buffer [PBS with 0.1% bovine serum albumin (BSA) and 2 mM EDTA] and CD4⁺ cells were removed using Mouse Depletion Dynabeads®. Bead-free CD4⁺ T cells were resuspended in 250 μ l isolation buffer, and 25 μ l FlowComp™ Mouse CD25 antibody and 75 μ l FlowComp™ Dynabeads® (Invitrogen Dynal AS, Oslo, Norway) were added. The tube was placed in the magnet for a minimum of 2 min and the supernatant was carefully removed. The bead-bound cells were resuspended in isolation buffer and the tube was removed from the magnet. The bead-bound cells were then carefully resuspended in FlowComp™ Release Buffer (PBS with 0.1% BSA and 2 mM EDTA), mixed by gentle pipetting 10 times, and the tube was repositioned in the magnet for 2 min. The supernatant containing the bead-free CD4⁺CD25⁺ cells were transferred to a new tube and isolation buffer was added, followed by centrifugation for 8 min at 350g. The supernatants were discarded and the cell pellet containing the isolated mouse CD4⁺CD25⁺ regulatory T cells (Treg cells) were resuspended in culture medium (RPMI-1640) (35). To confirm Treg cells, the cells were stained with fluorescein isothiocyanate (FITC)-anti-CD25 and phycoerythrin-anti-Foxp3 for Treg cells (1:100 dilution) and analyzed with a FACSCalibur flow cytometer equipped with CellQuest software. Purity was more than 95%. The Treg cells were used to measure the expressions of Foxp3, TGF- β 1 and IL-10, other cytokines, mast cell activation and the dominance of Treg cells.

Total T and Treg cells isolated from spleens of OVA-challenged and irradiated mice were cultured for 3, 7 or 14 days, and the viability of both cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. Total and Treg cells isolated from spleens of normal mice received irradiation once daily for 3 consecutive days at the same dose as *in vivo*, cultured for 3, 7 or 14 days, and viability was determined (36) (Supplementary Information for MTT assay; <http://dx.doi.org/10.1667/RR3082.1.S3>).

Immunohistochemistry (IHC)

Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 3 μ m thick sections. Lung sections were deparaffinized with xylene and washed using various percentages of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Slides were then blocked with 1.5% BSA in PBS for 1 h. For immunofluorescence, a polyclonal primary antibody against Foxp3 or CCR4 (diluted to 1:25) (Cell Signaling Technology, Danvers, MA) was then applied and the slides were incubated at room temperature for 1 h. After washing in PBS, slides were treated with the corresponding FITC or TEXAS-Red conjugated anti-IgG for 1 h at room temperature. Wash steps between each stage were performed for 1–2 min. After washing in PBS, the slides were mounted using aqueous mounting medium and were examined using an LSM 5 EXCITER confocal microscope (Carl Zeiss, Oberkochen, Germany) (37). The degree of IHC color (yellow) developed by co-localization was quantified by intensity in 200 $\times$ 200 μ m areas under microscope (5 areas/each slide $\times$ 8 mice/each group =

40 areas), and then mean $\pm$ SEM for 40 areas was presented by a histogram.

Preparation and Activation of Bone Marrow-Derived Mast Cells (BMMCs)

Bone marrow cells flushed from femurs and tibias of female, 8-week-old C57BL/6 mice were cultured and confirmed in RPMI-1640 supplemented 50% WEHI-3B conditioned media for 5 weeks (31). BMMCs (1×10^6 cells) were sensitized and challenged with anti-DNP IgE antibody (0.1 μ g/ml) and 1.0 μ g/ml dinitrophenol-horse serum albumin (DNP-HAS) for specified time periods at 37°C in Tyrode's buffer (32). Treg cells isolated from splenocytes were added to BMMCs 30 min before DNP-HSA stimulation. The supernatant and the cells were obtained by centrifugation at 400g for 5 min (Supplementary Information; <http://dx.doi.org/10.1667/RR3082.1.S3>). In all experiments, optimal time and concentration were first determined in preliminary experiments.

Determination of Cytokines or Leukotrienes (LTs) by ELISA

Amounts of IL-10, transforming growth factor-beta 1 (TGF- β 1; total and active forms) released in Treg cells isolated and cultured for 24 h from spleens of OVA-challenged mice, and TNF- α or leukotrienes (LTs) released in homogenized supernatants of lung tissues of OVA-challenged mice or from act-BMMCs co-cultured with Treg cells were determined by ELISA kit (IL-10, TGF- β 1, TNF- α ; BD Biosciences, San Jose, CA) (LTs, Cayman Chemical, Ann Arbor, MI). The lowest detection limits for IL-10, TGF- β 1, TNF- α , or LTs were 4.0, 1.7–15.4, 1.07 and 7.8 pg/ml, respectively (Supplementary Information; <http://dx.doi.org/10.1667/RR3082.1.S3>) (31).

Statistical Analysis

Experimental data are presented as the mean $\pm$ SEM. Unpaired Student's *t* test was used to compare two groups. $P < 0.05$ were regarded as significant. The densitometry analysis of immunoblots, PCR and EMSA was performed with Quantity One version 4.6.3 (Bio-Rad, Hercules, CA) and are presented as the mean $\pm$ SEM ($n = 4$) obtained from the ratio of each band density versus those in control and loading control (not shown) of four independent experiments by histogram.

RESULTS

Effects of Repeated Low-Dose Irradiation on γ -H2AX Expression in Various Organ Tissues of OVA-Challenged Mice

To investigate whether repeated low-dose-irradiation damage of T cells, which have been shown to have increased radiosensitivity, or whether T cell subpopulations have different radiosensitivity, we first examined the viability of total T and Treg cells. We found that irradiation did not affect the viability of T and Treg cells isolated from spleens of OVA-challenged mice that were cultured for 3 days. However, the viability of both cell types was decreased at day 7 of culture ($P < 0.05$) (Supplementary Fig. 1B; <http://dx.doi.org/10.1667/RR3082.1.S1>), and was markedly decreased regardless of whether they were irradiated or not at day 14 of culture ($P < 0.01$). Irradiation also did not affect viability of total T cells or Treg cells isolated from normal spleen that were cultured for 3 days. After 7 days ($P < 0.05$) and 14 days ($P < 0.01$) of culture,

the viability of both cell types was decreased, regardless of whether or not they had been irradiated (Supplementary Fig. 1C; <http://dx.doi.org/10.1667/RR3082.1.S1>).

A recently developed biomarker for normal tissue toxicity induced by radiation, phosphorylated histone H2AX, has become a powerful tool to monitor DNA double-strand breaks (DSBs) (38). We observed that repeated low-doses of ionizing radiation did not alter the expression of γ -H2AX in major organs such as brain, lung and spleen of OVA-challenged mice (Fig. 1A). Therefore, the irradiated dose used did not influence cell death and residual DSBs at the times we assayed.

Effect of Irradiation on Inflammatory Cell Recruitment into BAL Fluid

We next examined the mechanism of action of irradiation in OVA-challenged allergic asthma in mice. Surprisingly, we observed that irradiated-OVA mice had significantly reduced levels of total inflammatory cells (from $26.2 \pm 1.55 \times 10^5$ to $15.1 \pm 1.23 \times 10^5$; $P < 0.001$) with significantly lower levels of eosinophils (from $12.9 \pm 1.46 \times 10^5$ to $5.3 \pm 0.50 \times 10^5$; $P < 0.001$), macrophages ($8.2 \pm 0.25 \times 10^5$ to $4.5 \pm 0.12 \times 10^5$; $P < 0.01$), lymphocytes (from $2.6 \pm 0.17 \times 10^5$ to $2.2 \pm 0.05 \times 10^5$; $P < 0.05$), and neutrophils (from $2.5 \pm 0.13 \times 10^5$ to $2.0 \pm 0.11 \times 10^5$; $P < 0.05$), which were increased in OVA mice ($P < 0.001$) versus Con or 2 Gy mice (Fig. 1B).

Effect of Irradiation on Histopathological Changes in Lung Tissue

Irradiated-OVA mice displayed reduced numbers of inflammatory cells ($P < 0.001$) that had infiltrated into the surrounding peribronchial and perivascular areas in lung tissue of OVA mice (Fig. 1C). However, no significant changes for these inflammatory cells were observed in the 2 Gy mice, therefore, we will not hereafter describe the effect of irradiation (total 2 Gy) exposure alone. The total lung inflammation scores for lung histology are shown in the histogram (Fig. 1C, right panel). This result was the same as those observed in experiments that were repeated five times.

Goblet cell hyperplasia and mucus overproduction are important features of bronchial asthma (3). OVA mice displayed increased numbers of goblet cells (evident as pink dots) containing mucus (Fig. 1D). The number of goblet cells in irradiated-OVA mice was significantly reduced by 45.6%, as determined by the percentage of PAS-positive cells/total cells (from 72.2% to 39.3%; $P < 0.001$). Irradiated-OVA mice also reduced collagen deposition in the surrounding peribronchial and perivascular areas of lung versus OVA mice (Fig. 1E). Lung fibrosis scores using the Ashcroft score method were increased in OVA mice (0.9 ± 0.35 to 4.1 ± 0.23 ; $P < 0.001$), while those of irradiated-OVA mice decreased by 31.7% (from 4.1 ± 0.23 to 2.8 ± 0.13 ; $P < 0.01$).

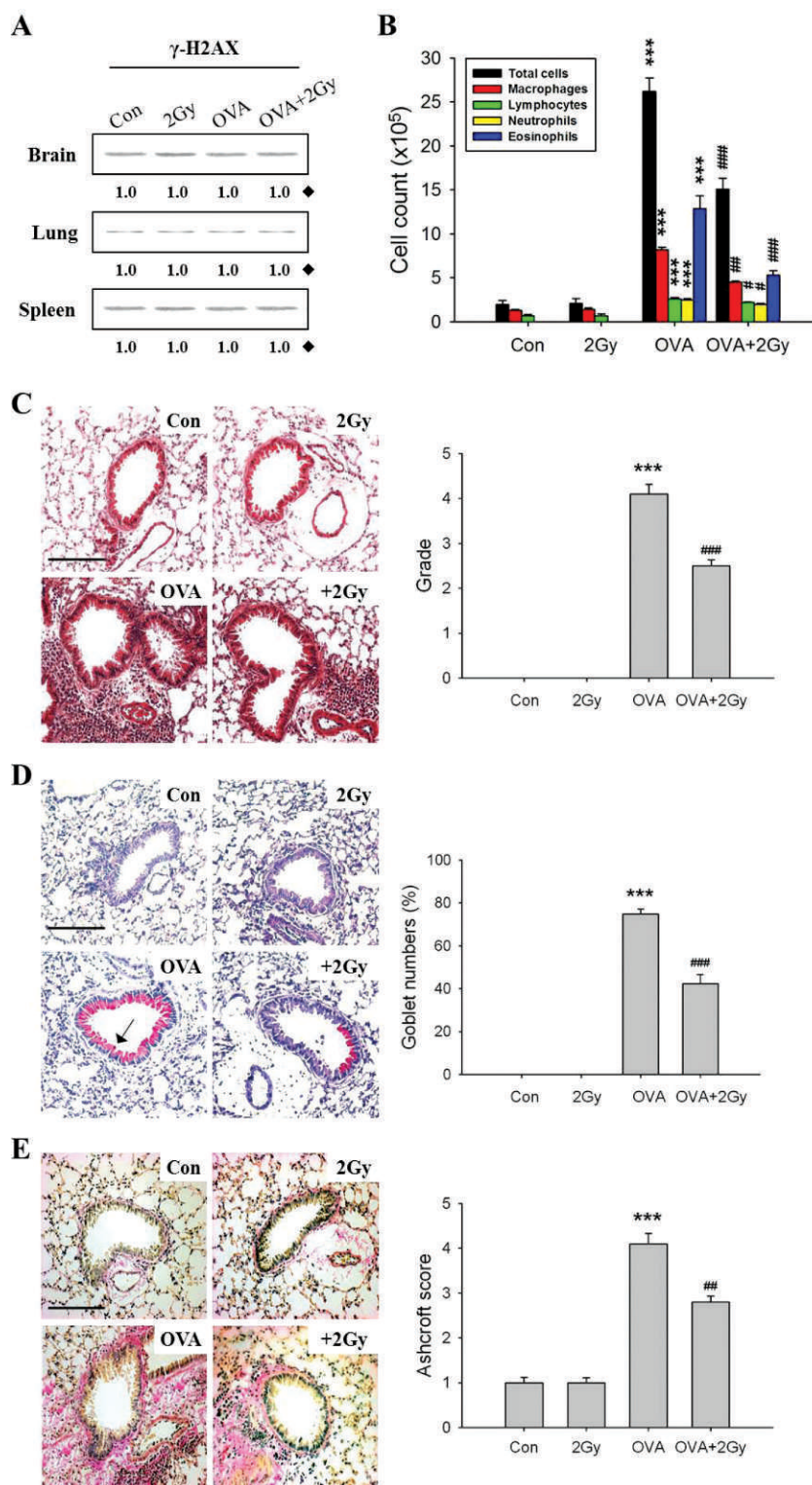


FIG. 1. Effects of repeated low-dose irradiation on γ -H2AX, inflammatory cells in BAL fluid, or histopathological changes in lung tissues of OVA-challenged mice. C57BL/6 mice were sensitized and challenged with OVA, and received whole-body irradiation without anesthesia once daily for 3 consecutive days at a dose of 0.667 Gy 24 h before every OVA or PBS challenge. Con (negative control), mice sensitized and nebulized with PBS; 2 Gy (irradiation alone), mice sensitized and nebulized with PBS and repeatedly exposed three times at a dose of 0.667 Gy (total dose, 2 Gy exposure); OVA, mice sensitized and nebulized with OVA; irradiated-OVA (+2 Gy), mice sensitized and nebulized with OVA and repeatedly exposed three times at a dose of 0.667 Gy. Panel A: Expression of γ -H2AX. Panel B: Number of inflammatory cells in BAL fluid. Panels C–E: Lung tissues stained with H&E, PAS and van Gieson, respectively. Histological appearances (magnification: 200 $\times$, right panel) were scored for the presence of peribronchial and perivascular inflammation. The scores were added together to give a total lung inflammation score (panel C), the ratio (%) of PAS-positive cells (indicated by the arrow)/total cells in 10 sites of 100 $\times$ 100 μ m area (panel D), and collagen deposition shown as histogram using the Ashcroft fibrosis score (panel E). Bars in the Con panels indicate 100 μ m. Data are expressed as the mean $\pm$ SEM ($n = 8$). *** $P < 0.001$ vs. Con or 2 Gy mice. * $P < 0.05$; ## $P < 0.01$; ### $P < 0.01$ vs. OVA mice.

Effect of Irradiation on EMBP and Muc5ac Expression and Collagen Production

Irradiation appeared to specifically reduce the recruitment of eosinophils (Fig. 1B). Expression of EMBP, which is overexpressed in eosinophilia (18, 39), was increased in OVA mice ($P < 0.001$) compared to that of control (Con) or 2 Gy mice. However, this increase was significantly reduced in BAL cells ($P < 0.05$) and lung tissue ($P < 0.01$) of the irradiated-OVA mice (Supplementary Fig. 2A; <http://dx.doi.org/10.1667/RR3082.1.S1>). Muc5ac is the mucus glycoprotein that is predominantly secreted during goblet cell hyperplasia in the respiratory tract of healthy and asthmatic individuals (18, 40). The expression of Muc5ac mRNA and protein was increased in BAL cells and lung tissue of OVA mice ($P < 0.001$) versus Con or 2 Gy mice, and reduced in irradiated-OVA mice ($P < 0.01$) (Supplementary Fig. 2B and C; <http://dx.doi.org/10.1667/RR3082.1.S1>).

To quantify lung fibrosis, collagen protein levels were measured using a Sircol assay. We found that collagen levels were significantly increased in the lung tissue of OVA mice ($P < 0.001$) versus Con or 2 Gy mice, but were reduced in irradiated-OVA ($P < 0.01$) (Supplementary Fig. 2D; <http://dx.doi.org/10.1667/RR3082.1.S1>).

Effects of Irradiation on Transcription Factor Activity and Expression of Cytokine mRNA

Migration of inflammatory cells from blood into the airway occurs through binding to specific adhesion molecules and chemokines (32), which are produced by the transcription factors NF- κ B and AP-1 in various diseases such as allergic airway disease (18, 40). Goblet cell hyperplasia is also produced by cytokines such as TNF- α , IL-1- β (33) and TGF- β (41). In our experiments irradiated-OVA mice displayed inhibited NF- κ B and AP-1 activities, which were increased in the BAL cells and lung tissues of OVA mice (Supplementary Fig. 3A; <http://dx.doi.org/10.1667/RR3082.1.S1>). Irradiated-OVA mice had reduced expression of various inflammatory cytokine mRNAs, which were increased in the BAL cells or lung tissues of OVA mice (Supplementary Fig. 3B, left and middle columns; <http://dx.doi.org/10.1667/RR3082.1.S1>). The data suggest that irradiation inhibits the recruitment of inflammatory cells into the airway system and also inhibits goblet hyperplasia via the inhibition of various cytokines and chemokines.

Effect of Irradiation on Serum OVA-Specific IgE Antibody Levels

Antigen-specific Th2 responses have been shown to induce antigen-specific antibody production (32). Therefore, we examined whether irradiation influenced OVA-specific IgE production in the allergic asthma mouse model (Supplementary Table 1, right column; <http://dx.doi.org/10.1667/RR3082.1.S2>).

Serum OVA-specific IgE antibody levels were elevated in OVA mice (238 ± 32.1 ng/ml; $P < 0.001$) compared to the Con mice (1 ± 0.4 ng/ml). Irradiated-OVA mice (175 ± 12.5 ng/ml) displayed 26.5% reduced OVA-specific IgE antibody levels ($P < 0.001$) versus OVA mice. However, irradiation exposure alone (total 2 Gy) did not affect IgE antibody production (2 ± 0.5 ng/ml). Thus, irradiation reduces the production of OVA-specific IgE antibody by regulating Th2 cytokines such as IL-4 and IL-13, which has been shown to be related to Ig class switching.

Effect of Irradiation on AHR

The above data supported anti-allergic activity of irradiation. To address this directly, AHR was assessed by changes in airway resistance (R_L) in response to increased doses of MCh. The R_L of OVA mice was markedly increased by MCh administration compared to Con mice ($P < 0.001$) (Fig. 2A). However, this increase was effectively reduced in irradiated-OVA mice ($P < 0.01$). The OVA mice (from 22.4 ± 1.42 to 6.7 ± 1.14 mg/ml) significantly decreased the concentration of MCh, which induced a 300% increase in the respiratory system (PC_{300}). Irradiated-OVA mice (15.8 ± 1.23 mg/ml) ($P < 0.01$) recovered PC_{300} compared to the OVA mice (Fig. 2B). However, exposure to irradiation alone (20.8 ± 0.71 mg/ml) did not affect the changes of R_L and PC_{300} versus Con mice (22.4 ± 0.63 mg/ml). The data implied that irradiation may reduce AHR by inhibiting Th2 cytokines in OVA-challenged mice.

Effect of Irradiation on the Changes of the Treg Cell Population

To clarify the mechanisms underlying the effects of irradiation, we first examined whether Treg cells were changed in irradiated-OVA mice. We found that irradiated-OVA mice ($5.2 \pm 0.32\%$ for BAL cells; $3.8 \pm 0.35\%$ for splenocytes, $P < 0.001$) displayed an increase in the Treg cell population that exceeded that of 2 Gy irradiated mice ($1.8 \pm 0.28\%$ for BAL cells, $P < 0.001$; $1.2 \pm 0.10\%$ for splenocytes, $P < 0.01$) (Fig. 3A) versus OVA mice ($1.0 \pm 0.15\%$ for BAL cells, $P < 0.01$; $0.7 \pm 0.12\%$ for splenocytes, $P < 0.05$) or Con ($0.3 \pm 0.23\%$ for BAL cells; $0.4 \pm 0.08\%$ for splenocytes). However, although the Treg cell population was increased in BAL fluid more than in the spleen, the total population of Treg cells in BAL fluid was too small to use for other experiments. Therefore, we used Treg cells isolated from spleen for this experiment.

As shown in Supplementary Fig. 1B and C (<http://dx.doi.org/10.1667/RR3082.1.S1>), the greater increment of Treg cells than of other T cells was not due to radioresistance. The population of Treg cells reached a maximum 3 days after irradiation (Supplementary Fig. 1D <http://dx.doi.org/10.1667/RR3082.1.S1>).

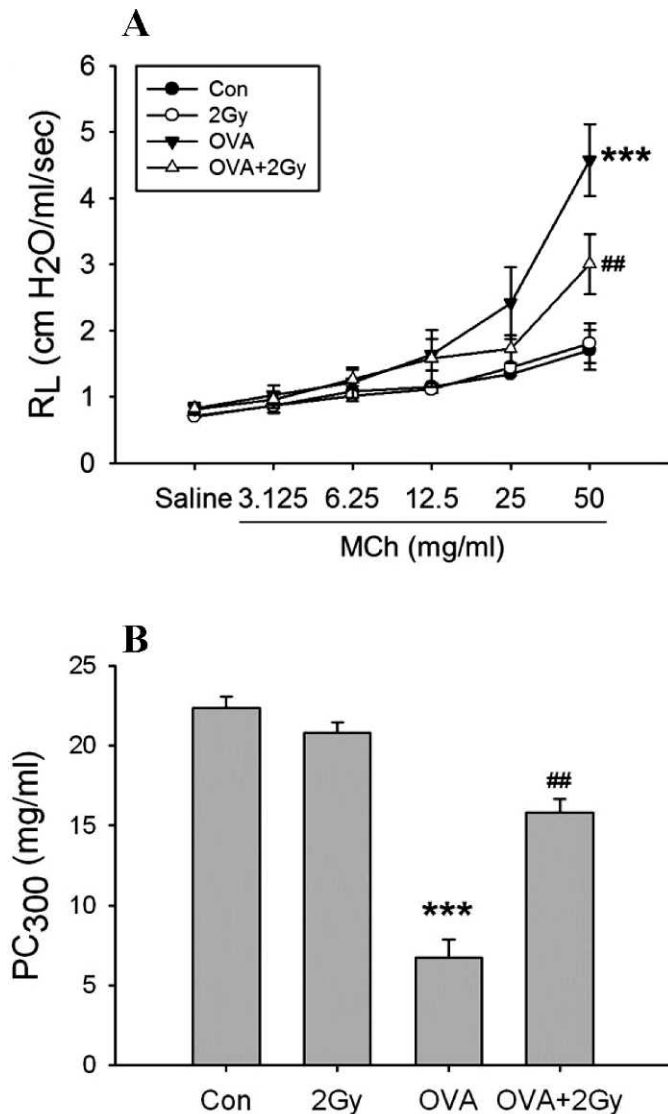


FIG. 2. Effect of repeated low-dose irradiation on airway hyperresponsiveness in OVA-challenged mice. Experimental details and all symbols used are described in the legend to Fig. 1. After each MCh nebulization, lung resistance was measured as cm H₂O/ml/s and as the percentage of the nebulized saline group. Provocative concentration (PC₃₀₀) was calculated by the dose of MCh that induced a 300% increase in R_L. Panel A: The lung resistance ($n = 8$). Panel B: PC₃₀₀ values. *** $P < 0.001$ vs. Con or 2 Gy mice. ## $P < 0.01$ vs. OVA mice. Data are expressed as the mean $\pm$ SEM ($n = 8$).

Effects of Irradiation on the Expression of Cytokines and Transcription Factors Related to Treg Cells

Treg cells isolated from spleens of irradiated-OVA mice enhanced mRNA and protein expression of Foxp3 ($P < 0.001$) (Fig. 3B), and IL-10 mRNA expression ($P < 0.001$) (Fig. 3C). The quantity of IL-10 protein secreted from Treg cells cultured for 24 h was enhanced (Table 1). In contrast, Treg cells from OVA mice displayed very markedly increased TGF- β 1 mRNA expression, while Treg cells from irradiated-OVA mice displayed reduced expression ($P < 0.01$), compared to the increase in OVA mice (Fig. 3C).

The amount of total and active TGF- β 1 protein that was secreted was also increased in Treg cell cultures of OVA mice ($P < 0.01$) versus those of Con mice (Table 1). The total TGF- β 1 protein level was also decreased in irradiated-OVA mice ($P < 0.05$). Furthermore, irradiated-OVA mice also displayed enhanced mRNA expression of IL-2, IL-10 and IL-35 related to the development of Treg cells in BAL cells ($P < 0.001$) and lung tissues ($P < 0.001$) versus those in OVA mice, but reduced TGF- β mRNA expression (Fig. 3D and E). Expressions of RUNX1 and NF- κ B were enhanced in Treg cells of irradiated-OVA mice ($P < 0.001$) versus OVA mice or irradiation alone (Fig. 3F). This data support that irradiation can change the Foxp3⁺-Treg cell population via RUNX1 and NF- κ B in OVA-challenged mice, and that Treg cells may have their suppressive activity via IL-10, not via TGF- β 1.

Effects of Irradiation on the Co-localization of Treg Cells and Chemokine Receptors

Since we observed the enhancement of Treg cells in spleens of irradiated-OVA mice, we next examined whether Treg cells were recruited and localized in lung tissues by measuring CCR4 expression, which is related to Treg cell recruitment. Irradiated-OVA (52 ± 6.4 cells) and 2 Gy irradiation alone (38 ± 5.5 cells, $P < 0.001$) (Fig. 4A, yellow color dot) displayed enhanced expression of Foxp3 (green color) and CCR4 expression (red color) in lung tissues versus OVA mice (6 ± 3.4 to 18 ± 4.2 cells, $P < 0.001$).

It has been suggested that the mechanism of action in Treg cells may involve secretion of IL-10/TGF- β and/or cell-cell contact (42, 43). However, IL-10 is not secreted in Treg cells only. This suggest that allergic responses are being attenuated via migration of Treg cells and imply that Foxp3⁺-Treg cells can migrate from other organs into lung tissues by irradiation.

Effects of Irradiation on The T Cell Subpopulation in Lung Tissue and Spleen

Pathogens or allergens have been shown to block *de novo* Treg cell differentiation and promote differentiation of effector/helper T cells (44), and Treg cells appear to suppress influx of effector T cells (activated-T cells, act-T cells). Therefore, we examined whether effector T cells (CD25) were increased in T cells activated with anti-CD3 antibody and anti-CD28 antibody (act-T cells) using T cells isolated from lung (0.3–38.1%) (Fig. 4B) and spleen (7.9–43.3%) (Fig. 4C) of OVA-challenged mice. We found that irradiated-OVA mice skewed act-T cells (surface marker, CD25) toward Treg cells (Foxp3; 2.6–14.1% for lung, 0.7–3.8% for spleen) versus those in OVA mice. Irradiated-/act-T cells isolated from spleen of normal mice also displayed skewed act-T cells toward Treg cells versus those in nonirradiated-/act-T cells (Fig. 4D). These data inferred that irradiation protects against the influx of act-T cells or blocks

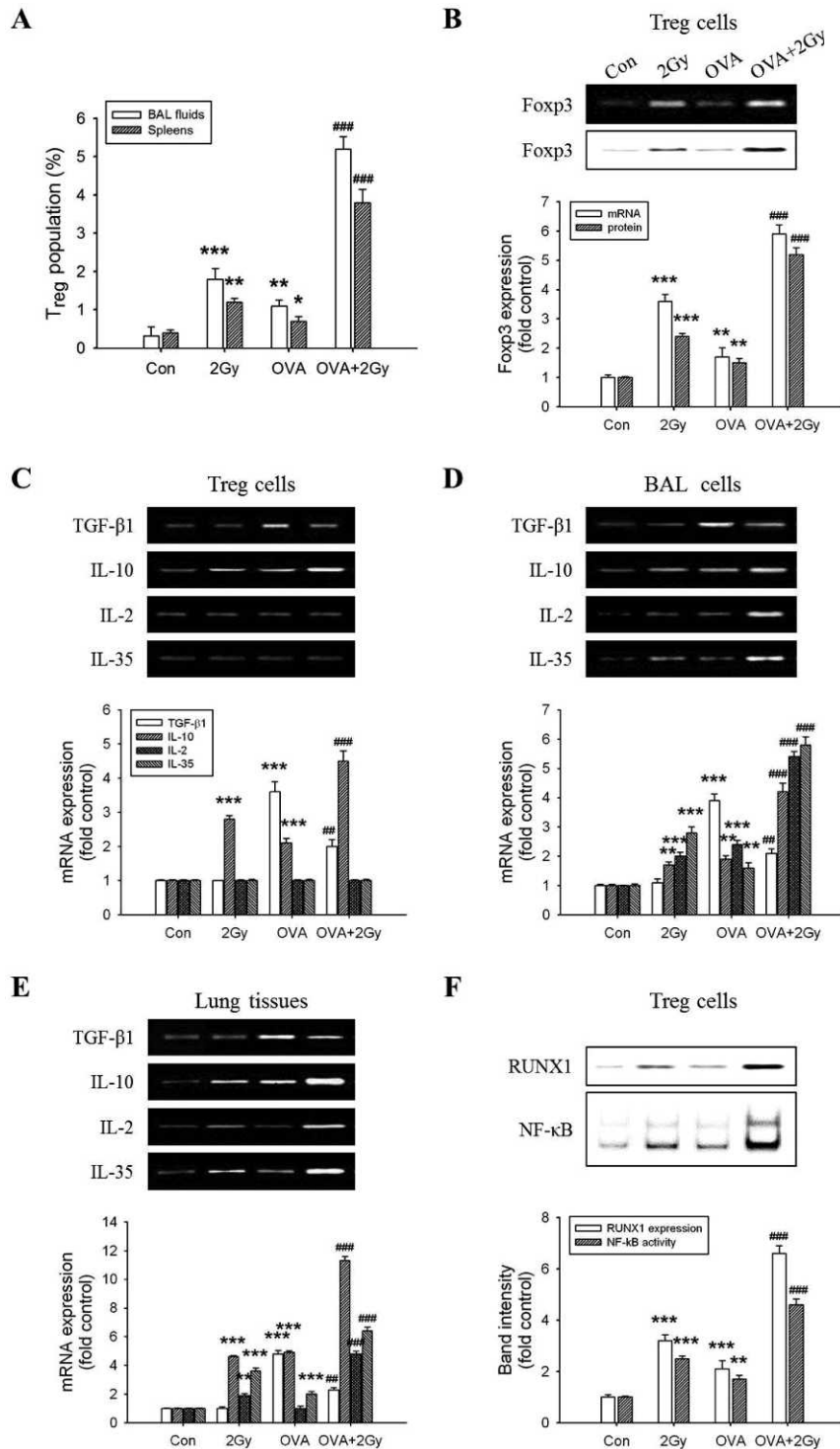


FIG. 3. Effect of repeated low-dose irradiation on number and expression of cytokines and transcription factors of Treg cells isolated and lung tissues from OVA-challenged mice. Experimental details and all symbols used were described in the legend to Fig. 1. Expression of Foxp3 protein and mRNA, individual cytokine mRNA and RUNX1, or NF- κ B activity were determined in protein, mRNA and nuclear extracts isolated from Treg cells, BAL cells, and lung tissue using Western blot, RT-PCR and EMSA, respectively. Panel A: Treg cell populations. The percentages represent the population of Treg cells out of 1×10^4 total cells. Panels B, C and F: Band images and histograms for expression of Foxp3, cytokine mRNA, and RUNX1 and NF- κ B activities in Treg cells, respectively. Panels D and E: Band images and histograms for expression of cytokine mRNA in BAL cells and lung tissue. The histogram from the densitometry analysis shows the mean $\pm$ SEM ($n = 4$) obtained from the ratio of each band density versus those of control and GAPDH or actin (band image not shown) from four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Con mice. ## $P < 0.01$; ### $P < 0.001$ vs. OVA mice.

the conversion of act-T cells. Lung tissues of irradiated-OVA mice also displayed increased Foxp3⁺ CD25⁺ cells that possessed the phenotype of Foxp3⁺ and IL-10⁺ cells, but not of Foxp3⁺ and IL-13⁺ cells (Supplementary Fig. 3C; <http://dx.doi.org/10.1667/RR3082.1.S1>).

Effects of Irradiation on Mast Cell Population and Expression of Surface Markers

Irradiated-OVA mice displayed reduced numbers of mast cells in BAL cells and lung tissues ($P < 0.01$) versus those increased in OVA mice (Fig. 5A). CCL2/CCR2 expression, which is associated with mast cell migration into pulmonary inflammatory sites (19), was increased in mast cells isolated from BAL cells or in lung tissue of OVA mice ($P < 0.001$), while mast cells or lung tissue of irradiated-OVA mice displayed reduced expression ($P < 0.001$) (Fig. 5B). As Treg cells suppress mast cell activation through OX40/OX40L interaction (27), we observed that Treg cells or mast cells isolated from BAL cells of OVA mice increased only the expression of OX40 and OX40L, respectively, ($P < 0.001$) versus those in Con mice (Fig. 5C). Treg cells from BAL cells of irradiated-OVA mice enhanced OX40 expression ($P < 0.001$), but mast cells decreased OX40L expression ($P < 0.05$).

Effects of Treg Cells on BMMCs Activated with Antigen/Antibody Reaction (act-BMMCs)

To confirm *in vitro* the relationship between Treg cells and mast cells that are proposed to occur *in vivo*, OX40 expression was enhanced in act-BMMCs co-cultured with Treg cells ($P < 0.001$), but OX40L expression was decreased versus act-BMMCs co-cultured without Treg cells ($P < 0.05$) (Fig. 5D). Expression of IL-10 protein and mRNA was enhanced in act-BMMCs co-cultured with Treg cells ($P < 0.01$) versus those co-cultured without Treg cells. Also, expression of TGF- β protein and mRNA was decreased in act-BMMCs co-cultured with Treg cells versus those co-cultured without Treg cells ($P < 0.001$) (Fig. 5E).

The act-BMMCs co-cultured with Treg cells (Table 2) or lung tissue (Supplementary Table 1, middle and right columns; <http://dx.doi.org/10.1667/RR3082.1.S2>) reduced the release of TNF- α (granular mediator) and LTs (newly synthesized mediator) ($P < 0.001$) versus those increased without Treg cells. Expression of all cytokine mRNA was decreased in act-BMMCs co-cultured with Treg cells versus those increased without Treg cells (Supplementary Fig. 3B, right column, <http://dx.doi.org/10.1667/RR3082.1.S1>). The data suggest that act-BMMCs co-cultured with Treg cells enhance the expression of IL-10, but not of TGF- β 1, which has Treg cell-suppressive activity.

DISCUSSION

Low-dose γ -irradiation can ameliorate symptoms of various chronic diseases such as autoimmune diseases (1,

TABLE 1
Effects of Repeated Low-Dose Irradiation on the Cytokines in Treg Cells Isolated Form Spleen of OVA-Challenged Mice^a

Group	IL-10 (pg/ml)	TGF- β 1 (pg/ml)	
		Total	Active
Con	25 $\pm$ 1.6	29 $\pm$ 2.0	2 $\pm$ 0.4
2 Gy	138 $\pm$ 10.0	30 $\pm$ 1.8	2 $\pm$ 0.3
OVA	216 $\pm$ 12.2***	42 $\pm$ 2.2**	3 $\pm$ 0.3**
+2 Gy	362 $\pm$ 16.3###	31 $\pm$ 2.4 [#]	2 $\pm$ 0.3

Note. ** $P < 0.01$; *** $P < 0.001$ vs. the Con mice; [#] $P < 0.05$; ### $P < 0.001$ vs. the OVA mice.

^a C57BL/6 mice were sensitized and challenged with OVA, and received whole-body irradiation without anesthesia once daily for 3 consecutive days at a dose of 0.667 Gy 24 h before every OVA or PBS challenge as described in Materials and Methods section. Con (negative control), mice sensitized and nebulized with PBS; 2 Gy (irradiation alone), mice sensitized and nebulized with PBS and repeatedly exposed 3 times at a dose of 0.667 Gy (total dose, 2 Gy exposure); OVA, mice sensitized and nebulized with OVA; Irradiated-OVA (+2 Gy), mice sensitized and nebulized with OVA and repeatedly exposed 3 times at a dose of 0.667 Gy. Treg isolation was described in Materials and Methods section ($n = 8$). Treg cells (1×10^6 cells) isolated from each group were determined amounts of IL-10, TGF- β 1 by ELISA.

4–6). However, its effect on allergies has remained unclear, with the exception of a reported exacerbation of asthma (12).

We demonstrate here that Foxp3⁺-Treg enhanced migration induced by irradiation reduced mast cell migration via the inhibition of cytokines and the CCL2/CCR2 axis, and reduced airway inflammation and remodeling in the allergic asthma model by inactivating mast cells via OX40/OX40L interaction. The fraction 2 Gy dose of irradiation was not toxic to brain, lung and spleen tissue, or to total T and Treg cells isolated from splenocytes of OVA-challenged mice or from splenocytes of normal mice (Supplementary Fig. 1B and C; <http://dx.doi.org/10.1667/RR3082.1.S1>), although higher doses of irradiation can be toxic.

Treg cell expression of the transcription factor Foxp3 has been shown to play an essential role in controlling immune responses to allergens. IL-2 regulates expression of Foxp3 in Treg cells (45), and the inhibitory cytokine IL-35 promotes Treg cell suppressive function (46, 47). In addition, expression of Foxp3 is also regulated by multiple transcription factors such as NFAT, AP-1, STAT5, CREB and NF- κ B, which modulate Treg cell development (20, 31, 30), and RUNXs, which control the induction and suppressive function of Treg cells (20, 25, 29, 30). Furthermore, Treg cells were shown to directly inhibit the activation of allergic-specific Th2 cells associated with the effector phase of allergic reactions (27), and the predominant inhibitory mechanism in Treg cells appears to involve anti-inflammatory cytokines IL-10 and TGF- β (23). Finally, data suggest that irradiation enhances Treg cells in the periphery (9, 48). In this context, our data suggest that irradiation enhances the Foxp3⁺-Treg cell population through regulation by NF- κ B/RUNX1, and that Treg cells

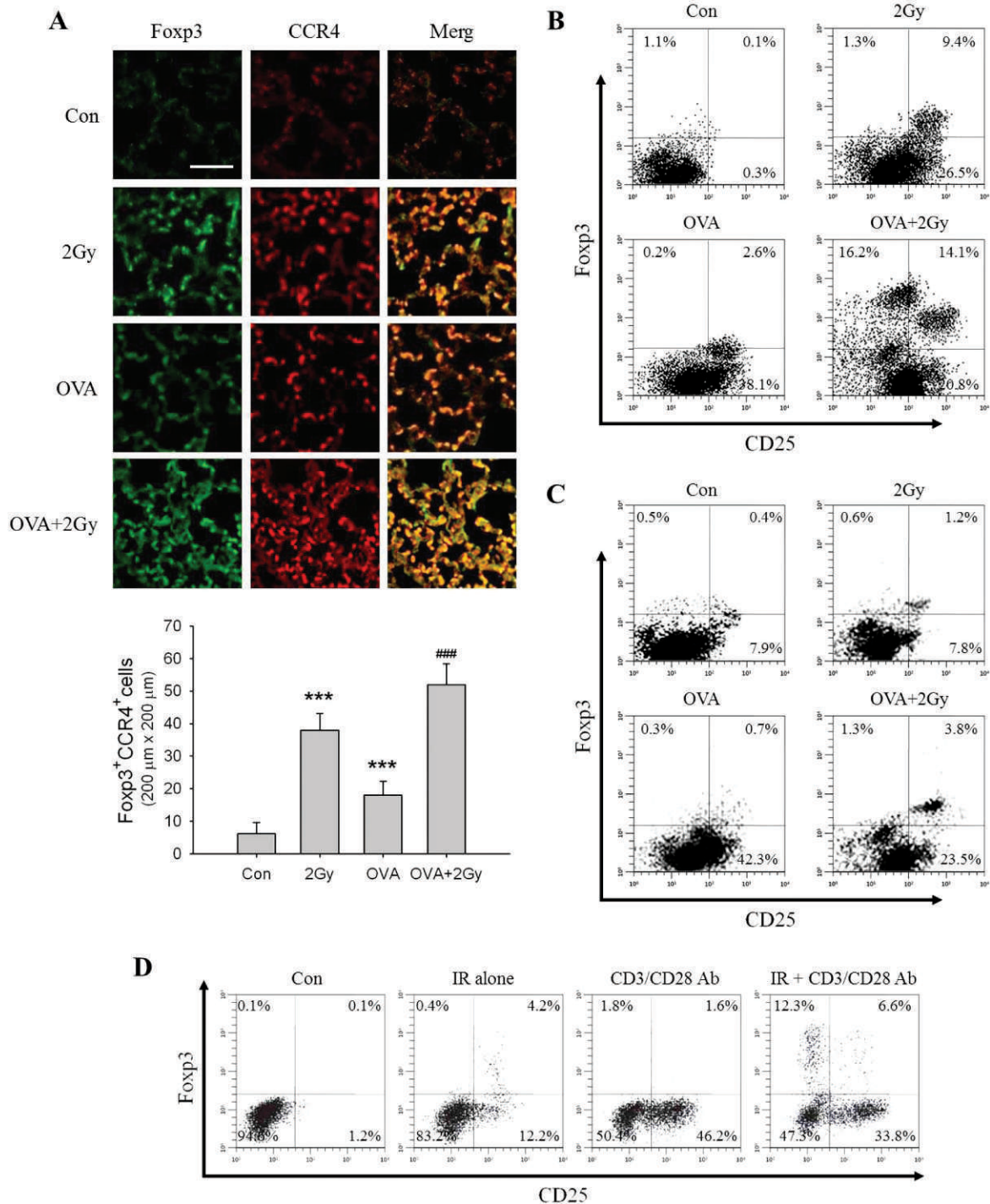


FIG. 4. Effects of repeated low-dose irradiation on the co-localization of Treg cells and CCR4 or the Treg cell population in the lung tissue and spleen of OVA-challenged mice, or in Treg cells isolated from spleen in normal mice. Experimental details and all symbols used were described in the legend to Fig. 1. Co-localization (upper right panel) of Treg cells and CCR4 in the lung tissues of OVA-challenged mice was determined by immunohistochemistry. The degree of antibody binding (yellow) indicating co-localization was quantified in a 200 × 200 μm field (5 areas/slide × 8 mice/group = 40 areas) and are depicted in a histogram. Treg cells and act-T cells were analyzed using flow cytometry. Panel A: Co-localization of Treg cells and CCR4 in the lung tissues. The white bar in the Con represents 100 μm. *** $P < 0.001$ vs. Con mice. ### $P < 0.001$ vs. OVA mice. Panels B and C: Population of Treg cells (Foxp3) and act-T cells (CD25) included in total T cells isolated from lung tissues (panel B) and spleen (panel C) of OVA-challenged mice. Panel D: Population of Treg cells (Foxp3) and act-T cells (CD25) in T cells isolated, irradiated and activated from normal mouse spleen.

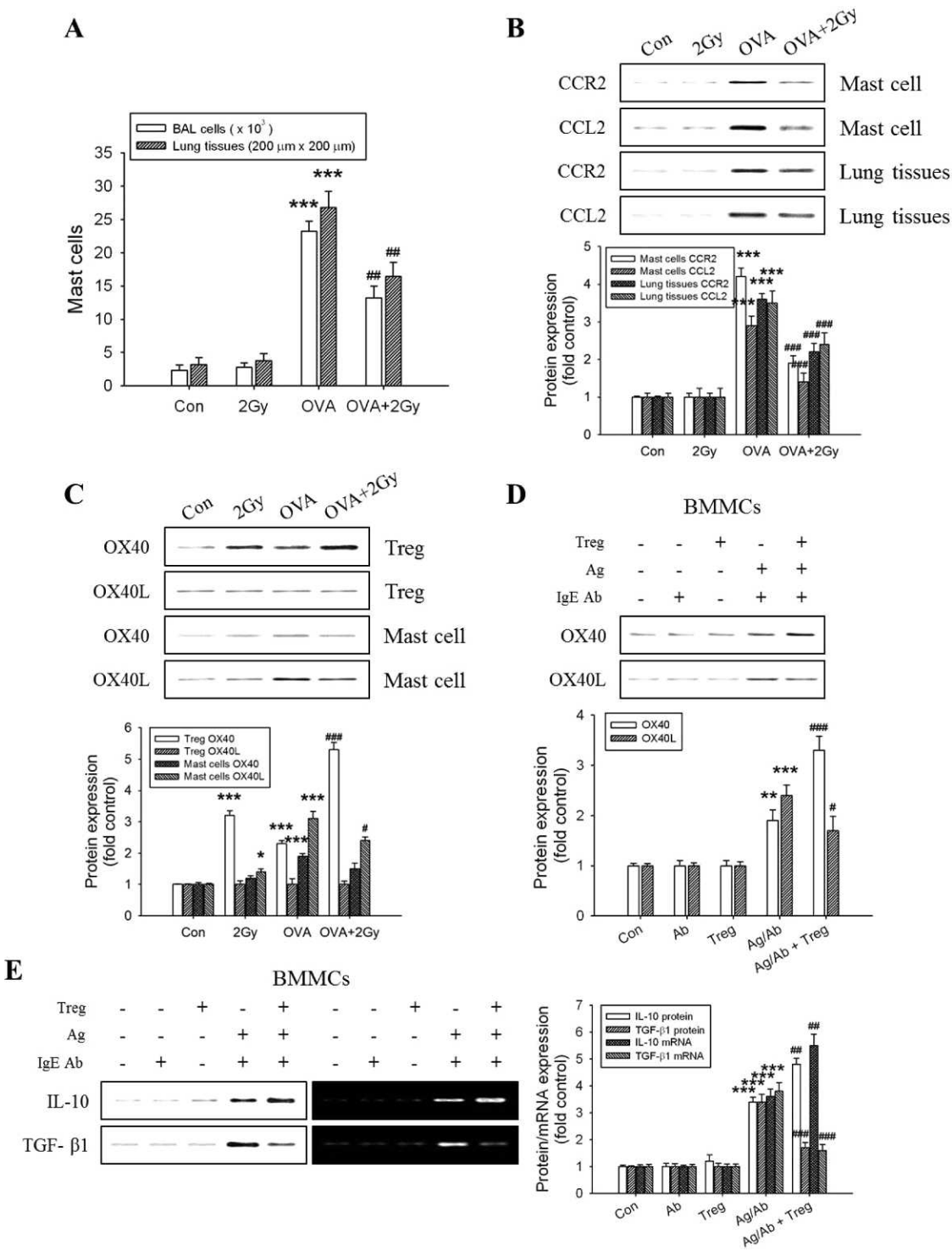


FIG. 5. Effects of repeated low-dose irradiation on the number, surface markers and cytokine expression of mast cells with Treg *in vivo* and *in vitro*. Experimental details and all symbols used were described in the legend to Fig. 1. Mast cells were quantified and Treg cells were isolated, as described in Materials and Methods. Expression of CCL2/CCR2, OX40/OX40L, IL-10 and TGF- β were determined in protein and mRNA extracts isolated from BMMCs or BAL cells and lung tissues of OVA-challenged mice using Western blot and RT-PCR, respectively. Panel A: Mast cell population in lung tissues of OVA-challenged mice. Panel B: Expression of CCL2/CCR2 in mast cells isolated from BAL cells and lung tissue of OVA-challenged mice. Panel C: Expression of OX40/OX40L in Treg cells and mast cells isolated from BAL cells of OVA mice. Panel D: Expression of OX40/OX40L in BMMCs co-cultured with Treg cells. Panel E: Expression of IL-10 and TGF- β in act-BMMCs co-cultured with Treg cells, (-) or (+), without and with materials. The histogram from the densitometry analysis was obtained, as described in the legend to Fig. 3 ($n = 4$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Con mice or act-BMMCs without Treg cells. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. OVA mice or act-BMMCs co-cultured with Treg cells.

TABLE 2
Effects of Repeated Low-Dose Irradiation on the Cytokines in
BMMCs Activated with Antigen/Antibody Reaction^a

Group	TNF- α (pg/ml)	LTs (pg/ml)
BM	88 $\pm$ 10.4	132 $\pm$ 11.8
Treg	82 $\pm$ 10.8	126 $\pm$ 12.4
Ag/Ab Rx	840 $\pm$ 42.8***	4221 $\pm$ 126.1***
+ Treg	138 $\pm$ 22.2###	1623 $\pm$ 61.2###

Note. *** $P < 0.001$ vs. BM; ### $P < 0.001$ vs. Ag/Ab Rx.

^a BMMCs were isolated as described in Materials and Methods section ($n = 8$). BMMCs (1×10^6 cells) were stimulated with anti-DNP IgE Ab/DNP-HSA and Treg cells (1×10^6 cells). Amounts of TNF- α , LTs were determined by ELISA. BM, un-act-BMMCs; Treg, Treg cells alone; Ag/Ab Rx, DNP-HSA/anti-DNP IgE antibody reaction; Ag/Ab Rx + Treg, act-BMMCs with Treg.

may have a suppressive function via IL-10, but not via TGF- β , in lung tissues of OVA-challenged mice.

It has been reported that irradiation enhances Treg cells (9) and that various cytokines convert naïve CD4⁺CD25⁻ cells to CD4⁺Foxp3⁺ Treg cells (49, 50). Therefore, we propose that the increase of Treg cells after irradiation exposure alone is solely due to the effects of radiation, and that the increase in Treg cells observed in irradiated-OVA mice is due to both the effects of radiation and the cytokines produced by OVA. Foxp3⁺ transcripts were enhanced in irradiated-OVA mice more than in mice solely irradiated with a dose of 2 Gy.

Our data also indicates that Treg cell enhancement by irradiation may be related to the reduced infiltration of eosinophils. This is further supported by the inhibition of eosinophil numbers and granular EMBP expression (Supplementary Fig. 2A; <http://dx.doi.org/10.1667/RR3082.1.S1>) after radiation exposure, which are markers of eosinophil numbers and elevated eosinophil secondary granules, respectively, in asthmatic patients. However, our data could not conclusively demonstrate whether irradiation itself reduced the infiltration of eosinophils (Fig. 1B) by inhibiting the expression of IL-5 (Fig. 1C) or chemokines such as IL-8 produced by NF- κ B activation (18) (Supplementary Fig. 3A and B; <http://dx.doi.org/10.1667/RR3082.1.S1>). It also remains unclear whether Treg cells migrating from other organs such as the spleen reduce inflammatory cell recruitment and production of cytokines.

Allergen-induced AHR is regarded as a manifestation of a Th2-dependent allergic disorder. Th2-associated cytokines, such as IL-4, IL-5, IL-8, IL-13 and TNF- α , are the key cytokines involved in the induction of AHR, eosinophil infiltration into the lung, mucus secretion (18, 32), and production of collagen (51). Therefore, changes in airway remodeling, which occur in response to persistent inflammation, are correlated with the development of AHR (18, 52). Airway epithelial NF- κ B activation has also been shown to enhance the production of inflammatory cytokines and AHR and mucus secretion in mouse allergic inflammation (53, 54). Moreover, Treg cells appear to directly

inhibit the activation of allergic-specific Th2 cells, and thereby minimize the production of the IL-4, IL-5, IL-8 and IL-13 cytokines that are essential during the effector phase of allergic reactions (55) resulting in the suppression of the allergic inflammation through direct action on inflammatory cells (21, 28). Therefore, our data suggest that irradiation inhibits AHR by reduction of cytokines as a result of the suppressive activity of Treg cells recruited by chemokines, and/or a reduction of allergic inflammation and airway remodeling through inhibition of Th2-associated cytokines by NF- κ B and AP-1 activation.

B cells require cytokines, such as IL-4 and IL-13, and CD40 ligand to switch to IgE production (18, 56). IgE plays a pivotal role in the development of allergic asthma (57). Treg cells can exert a direct effect on B cells by suppressing the production of allergen-specific IgE (22, 25, 58). Our data suggest that Treg cells enhanced by irradiation may inhibit the production of IgE (Supplementary Table 1; <http://dx.doi.org/10.1667/RR3082.1.S2>) by IL-4 and IL-13 (Supplementary Fig. 3B; <http://dx.doi.org/10.1667/RR3082.1.S1>), although the involvement of CD40 ligand was not examined.

TGF- β is a pleiotropic cytokine that regulates lymphocyte homeostasis and has been implicated in suppressing Treg cells that control allergic airway diseases (58, 59). However, other studies have reported a central role of IL-10 with or without TGF- β in controlling allergic airway inflammation (23, 60, 61). TGF- β also has been shown to play an important role in promoting the structural changes in tissue remodeling such as induction of collagen expression and enhancement of goblet cell proliferation (41). Our data suggest irradiation can reduce OVA-induced allergic asthma symptoms through the inhibition of TGF- β . This proposal is supported by our observation that irradiation enhances Foxp3⁺ and IL-10-producing Treg cells, and thereby leads to the inhibition of TGF- β by the elevation of Treg cells (Fig. 3C–E). The increased expression of TGF- β in Treg cells from the spleen or in BAL cells and lung tissues of OVA support our view that it may be produced in the various inflammatory cells that are recruited through cytokines produced by antigen (OVA). This requires further study.

Since Treg cells may exert their suppressive activity by cell-cell contact as well as through cytokine secretion, the appropriate localization of Treg cells is important to determine its function (7). Recruitment of Treg cells into the lung is impaired in the absence of CCR4 (62), and we observed the co-localization of Treg (Foxp3) and CCR4, which is required for the recruitment of Treg cells to the lung tissues (Fig. 4A). This data implies that Treg cells after irradiation migrate into the lung from other organs such as the spleen. However, we do not know whether the Treg cells increase in the lung tissues after irradiation are due to migration from resident cells of other organs or are due to migration from induced cells. Further study is required.

Mature mast cells residing in the lung have been shown to counteract Treg cells through IL-6 and/or the OX40/OX40L axis (27, 28). However, Treg cells, in the presence TGF- β

and IL-2 have also been shown to promote the recruitment of mast cell progenitors (MCP) to the lung with antigen-induced pulmonary inflammation (63). In our study, the population of mast cells was reduced in irradiated-OVA mice. Therefore, the data suggest that Treg cells enhancement by irradiation may reduce the migration of mast cells into lung tissues through inhibition of the expression of cytokines and CCL2/CCR2, and that Treg cells may inhibit mast cell activation by OX40/OX40L interaction, followed by the inhibition of mediator release (TNF- α , LTs and cytokines). However, further study is necessary to investigate the MCP migration into lung tissues.

In conclusion, our data suggest that Treg cells enhancement/migration by irradiation exert a suppressive function in airway inflammation and tissue remodeling in allergic asthma through the inhibition of mast cell migration into the lung by cytokines and the CCL2/CCR2 axis, and through the inactivation of mast cells by cell-cell contact, such as OX40/OX40L, followed by the release of mediators, such as TNF- α , LTs and inflammatory cytokines. However, the possibility that irradiation itself may inhibit Th2-cytokine production by regulating the recruitment of inflammatory cells and thereby suppress airway allergic responses cannot be excluded. Our data are unlikely to be directly applied to human therapy because whole-body irradiation of mice was used in this study. However, our study does at least suggest the possibility that irradiation can be used for the reduction of inflammation in patients with chronic diseases with allergic asthma such as mastocytosis. Additional research needs to investigate whether Treg cells enhanced in lung tissues by irradiation are migrated by the resident Treg cells or the induced-Treg cells.

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