

Detection of Allergenic Compounds Using an IL-4/Luciferase/CNS-1 Transgenic Mice Model

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The interleukin-4 (IL-4) signaling cascade has been identified as a potentially important pathway in the development of allergies. The principal objective of this study was to produce novel transgenic (Tg) mice harboring the luciferase gene under the control of the human IL-4 promoter and the enhancer of IL-4 (CNS-1), in an effort to evaluate three types of allergens including a respiratory sensitizer, vaccine additives, and crude extracts of natural allergens *in vivo*. A new lineage of Tg mice was generated by the microinjection of *pIL-4/Luc/CNS-1* constructs into a fertilized mice egg. The luciferase activity was successfully regulated by the IL-4 promoter in splenocytes cultured from IL-4/Luc/CNS-1 Tg mice. From the first five founder lines, one (#57) evidencing a profound response to ovalbumin was selected for use in evaluating the allergens. Additionally, the lungs, thymus, and lymph nodes of IL-4/Luc/CNS-1 Tg mice evidenced high luciferase activity in response to allergens such as phthalic anhydride (PA), trimellitic anhydride, ovalbumin, gelatin, *Dermatophagoides pteronyssinus* extracts, and Japanese cedar pollen, whereas key allergy-related indicators including ear thickness, Immunoglobulin E concentration, and the infiltration of inflammatory leukocytes in response to PA were unaltered in the Tg mice relative to the non-Tg mice. Furthermore, the expression levels of endogenous type 2 helper T cells cytokines and proinflammatory cytokines were similarly increased in these organs of IL-4/Luc/CNS-1 Tg mice in response to allergens. These results indicate that IL-4/Luc/CNS-1 Tg mice may be used as an animal model for the evaluation and prediction of the human body response to a variety of allergens originating from the environment and from certain industrial products.

Key Words: allergens; IL-4; transgenic mice; luciferase; evaluation.

With the proliferation of allergens in industrialized countries, such as pollution and food additives, the incidence of allergic diseases such as atopic dermatitis, asthma, and rhinitis has

increased significantly (Gizzarelli *et al.*, 2006; Randhawa and Bahna, 2009). Recently, several traditional methods are being used in the clinical context to screen allergens among substances originating from the environment. Among these methods, the allergy skin prick test is a cheap, rapid, and accurate method, in which a few drops of isolated allergen are gently pricked onto the surface of the forearm skin in order to identify the causative allergens (Turner *et al.*, 1980). However, this method is limited in that it is difficult to evaluate quantitatively the magnitude of the allergic response. Another method, the allergy-specific Immunoglobulin E (IgE) antibody test, screens allergens by measuring blood samples for the amounts of the Immunoglobulin E (IgE) antibody for the suspected allergens (Johansson, 2004; Petersen *et al.*, 2004). However, it is also limited in that levels of IgE antibodies against allergens in blood do not always correlate with the allergic reaction.

Some transgenic (Tg) animal models featuring an interleukin-4 (IL-4)-regulated reporter gene have been developed for assessing immunity *in vivo* (Mohrs *et al.*, 2001). In this study, we develop a tool using Tg mice expressing the luciferase gene under the control of the human IL-4 promoter and an IL-4 enhancer (conserved noncoding sequence-1, CNS-1) in order to detect allergens in the environment effectively. CNS-1 was mapped within the *IL-4/IL-13* intergenic region of the type 2 helper T cells (Th2) cytokine locus and was identified as a potent enhancer of IL-4 promoter activity (Strempel and Vercelli, 2007). IL-4/Luc/CNS-1 Tg mice evidenced higher luciferase activity in immune-related organs such as the lymph node, thymus, and lung after treatment with allergens including respiratory sensitizer, vaccine additives, and crude extracts of natural allergens. These results indicate that the proposed allergen detection system using IL-4/Luc/CNS-1 Tg mice may facilitate the evaluation and prediction of human body responses against a variety of environmental allergens.

MATERIALS AND METHODS

Plasmid construction. *pIL-4/Luc/CNS-1* was constructed by cloning the human IL-4 promoter, as well as CNS-1 as the enhancer of the human IL-4 promoter, into *pTransLuciferase* (Panomics, CA) harboring the firefly luciferase gene (Fig. 1A). The human IL-4 promoter (-1169 to -172, relative to the IL-4 ATG; GenBank accession No. M23442) was amplified via DNA-PCR using genomic DNA. *pIL-4/Luc* was generated by cloning the 998-bp products of the amplified human IL-4 sequence into the *NheI* and *BglI* restriction sites of the *pTransLuciferase* vector. CNS-1 (GenBank accession No. AC004039; nucleotides 42,293–42,810) was also amplified via DNA-PCR using genomic DNA. *pIL-4/Luc/CNS-1* was generated by cloning the 518-bp products of the amplified CNS-1 sequence into the *SaI* restriction sites of *pIL-4/Luc*.

Production and PCR genotyping of Tg mice. The *pIL-4/Luc/CNS-1* plasmid was digested with *BglI* in order to remove the prokaryotic sequence and subsequently microinjected into the male pronuclei of fertilized embryos, which had been previously obtained by crossing C57BL/6 (female) mice with DBA/2 (male) mice. The injected eggs were then transferred into the oviducts of a pseudopregnant ICR recipient female. The transgene was identified via DNA-PCR analysis of the genomic DNA isolated from the tails of the 3-week-old founder mice. After amplification, the product (467 bp) levels were quantified on 1% agarose gel.

Splenocyte cultures. Spleens of IL-4/Luc/CNS-1 Tg mice were aseptically collected. Single-splenocyte suspensions were prepared by lysing the erythrocytes with RBC lysis solution (Qiagen, MD) and cultured in a complete RPMI-1640 medium (Invitrogen, CA). Spleen cells (1×10^6) from different groups were added at 10 ml per well of culture plate (Corning, NY). Splenocytes of IL-4/Luc/CNS-1 Tg mice and non-Tg mice were stimulated with ovalbumin at varying times and concentrations.

Measurement of ear thickness. Ear thickness was measured to determine the degree of allergic skin inflammation induced by phthalic anhydride (PA) treatment. In brief, a thickness gauge (Digimatic Indicator, Matusutoyo Co., Tokyo, Japan) was used to measure the thickness of the ear. The increase in ear thickness was then determined by subtracting the ear thickness prior to the first treatment.

Histological analysis. The ear skin samples removed from the mice were fixed in 10% formalin, embedded in paraffin wax, and subsequently sectioned into 5- μ m-thick slices. The skin sections were then stained with hematoxylin and eosin or toluidine blue (Sigma-Aldrich, MO). The stained skin sections were examined under light microscopy for the presence of edema and accumulation of inflammatory cells.

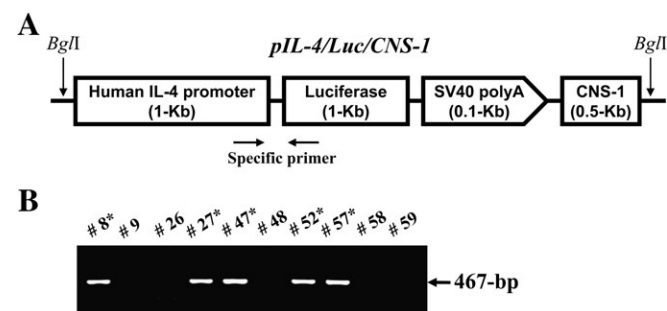


FIG. 1. Construction of the *pIL-4/Luc/CNS-1* reporter vector and identification of the IL-4/Luc/CNS-1 Tg using PCR. (A) *pIL-4/Luc/CNS-1* was constructed by cloning the human IL-4 promoter and CNS-1 as an enhancer of human IL-4 promoter into *TransLuciferase* containing the firefly luciferase gene. (B) DNA-PCR was conducted on genomic DNA isolated from the tails of founder mice.

Myeloperoxidase activity in inflamed tissue. The ears were homogenized in 1 ml of tissue protein extraction reagent (Pierce, IL) containing a protease inhibitor cocktail. The homogenates were subsequently centrifuged for 30 min at $16,000 \times g$, after which the MPO activity was assessed using an ELISA kit (Hycult Biotechnology, Uden, The Netherlands) in accordance with the manufacturer's instructions. The protein content of the homogenates was also measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories) in accordance with the manufacturer's instructions, after which the MPO activity was normalized on the basis of the protein content.

Luciferase activity assay. The activity level of luciferase was detected via the *in vitro* quantitative assay procedures and reagents of a Luciferase Reporter Assay Kit (Promega, WI), in accordance with the manufacturer's instructions. The protein concentration for each lysate was quantified using a bicinchoninic acid protein assay (Thermo Scientific, IL). The relative luciferase activity for each sample represents luciferase counts corrected for total protein content.

Allergen treatments. First, 8-week-old mice were divided randomly into three subgroups ($n = 5$) per group. For the first subgroup (designated acetone-olive oil [AOO]), 100 μ l of acetone-olive oil was repeatedly spread on each ear and the shaved dorsal region of each experimental mouse three times a week for 3 weeks. For the second and third subgroups (designated as 5% PA and 10% trimellitic anhydride [TMA], respectively), 100 μ l of 5% PA solution or 100 μ l of 10% TMA solution in vehicle (4:1 for acetone-olive oil, vol/vol) was sprayed repeatedly on each ear and the shaved dorsal region of each mice three times a week for 3 weeks. To administer the ovalbumin and gelatin, the 8-week-old mice were divided randomly into three subgroups ($n = 5$) per group. For the first subgroup, mice were injected ip with 1 mg/kg/day of ovalbumin (Sigma-Aldrich) in 0.9% saline three times per week for 3 weeks. For the second subgroup, the mice were injected ip with 1 mg/kg/day of gelatin (Sigma-Aldrich) in 0.9% saline three times a week for 3 weeks. For the third subgroup, the mice were injected ip with 0.9% saline three times a week for 3 weeks. To administer the *Dermatophagoides pteronyssinus* extracts (DPE), 8-week-old mice were divided randomly into two subgroups ($n = 5$) per group. For topical application, the shaved dorsal regions of Tg mice were treated with commercially available DPE (Biostir-AD, Biostir, Kobe, Japan) according to the methods suggested by Jung *et al.*, (2010). For the first subgroup, 100 mg of Biostir-AD was applied to the shaved dorsal skin twice per week for 3 weeks. The second subgroup was treated with vehicle solution only for 3 weeks. To administer Japanese cedar pollen (JCP) or cockroach extract (CE), Tg mice (five per group) were sensitized via intranasal administration of either 5 μ g of JCP or 5 μ g of CE in 10 μ l of PBS without adjuvants once per week for 3 weeks (on days 1, 8, 15, and 22). After the fourth sensitization, mice were challenged via intranasal administration every day for 6 consecutive days (on days 23–27) (See supplementary data). JCP and CE were purchased from the Biostir Company.

Bioluminescence imaging and data analysis. *In vivo* imaging was conducted using an IVIS imaging system (Xenogen, CA) as described previously. IL-4/Luc/CNS-1 Tg mice were anesthetized with Zoletil and injected ip with 150 mg/kg of D-luciferin (Sigma-Aldrich). Ten minutes after D-luciferin injection, images of mice were taken for 3 min using an IVIS imaging system. Photons emitted from specific regions were quantified using Living-Image software (Xenogen). *In vivo* luciferase activity was expressed in photons per second.

Semiquantitative RT-PCR analysis of cytokine gene expression. reverse transcription (RT)-PCR was conducted to measure the relative quantities of messenger RNA (mRNA) for IL-4, IL-5, IL-13, and tumor necrosis factor (TNF)- α in eight organs of IL-4/Luc/CNS-1 Tg mice and non-Tg mice. For RT-PCR analysis, each tissue sample was frozen in liquid nitrogen. The frozen tissues were chopped with scissors and homogenized in an RNazol B solution (Tet-Test Inc., TX). The isolated RNA was then determined via UV spectroscopy. The expression of the transgenes was assessed using RT-PCR with 5 μ g of the total RNA from each tissue. Five hundred nanograms of the oligo-dT primer (Invitrogen) was annealed at 70°C for 10 min. The complementary DNA, which was used as the template for further amplification,

was synthesized via the addition of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate with 200 units of reverse transcriptase. Ten picomole of the sense and antisense primers were then added, and the reaction mixture was subjected to 35 cycles of amplification. Amplification was conducted in a Perkin-Elmer Thermal Cycler using the following cycles: 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. In each case, minus-RT controls were included in order to differentiate the DNA and RNA products. RT-PCR was also conducted using β -actin-specific primers in order to ensure RNA integrity. The primer sequences for cytokine expression identification were as follows: IL-4, 5'-GAA TGT ACC AGG AGC CAT ATC-3' and 5'-CTC AGT ACT ACG AGT AAT CCA-3'; IL-5, 5'-TCA CCG AGC TCT GTT GAC AA-3' and 5'-CCA CAC TTC TCT TTT TGG CG-3'; IL-13, 5'-TGC CAT CTA CAG GAC CCA GA-3' and 5'-CTC ACG GTC TTG CTG CAG TT-3'; and TNF- α , 5'-CCT GTA GCC CAC GTC GTA GC-3' and 5'-TTG ACC TCA GCG CTG AGT TG-3'. The sequences of the β -actin sense and antisense primers were 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3', respectively. The experiment was repeated three times. All samples were analyzed in triplicate. The final PCR products were separated on 2% agarose gel and then visualized via ethidium bromide staining.

Statistical analysis. One-way ANOVA was used to determine whether or not significant differences existed between the allergen-treated group and vehicle-treated group (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). Additionally, differences in the responses of IL-4/Luc/CNS-1 Tg mice and Non-Tg mice were evaluated via a *post hoc* test (SPSS for Windows, Release 10.10, Standard Version) of the variance and significance levels. All values were expressed as means $\pm$ SDs. A *p* value of < 0.05 was considered significant.

RESULTS

Production and Maintenance of Tg Mice

In order to generate the IL-4/Luc/CNS-1 Tg mice harboring the luciferase gene under the control of the IL-4 promoter and CNS-1 enhancer in each tissue, the IL-4/Luc/CNS-1 fragment (Fig. 1A) was microinjected into the male pronuclei of fertilized embryos. Among a total of 113 offspring (53 males and 60 females), two females (#27 and #57) and three males (#8, #47, and #52) from the first lineage founder mice harbored the IL-4/Luc/CNS-1 gene, as identified by genomic DNA-PCR (Fig. 1B). The founder mice harboring IL-4/Luc/CNS-1 were then backcrossed to the C57BL/6 background for three generations.

Selection of the IL-4/Luc/CNS-1 Tg Mice Evidencing a High Response to Ovalbumin

In order to determine whether the introduced luciferase gene was expressed quantitatively under the control of the IL-4 promoter and CNS-1 enhancer against allergens in tissues, the luciferase activity in splenocytes of IL-4/Luc/CNS-1 Tg mice and non-Tg mice was evaluated via a luciferase reporter assay after treatment with ovalbumin as an allergen. First, to determine the proper time and dose conditions for ovalbumin treatment, luciferase activity was measured in the splenocytes of IL-4/Luc/CNS-1 Tg mice and non-Tg mice under different conditions. These reporter assays demonstrated that luciferase activity was increased significantly with increasing ovalbumin concentrations, reaching a peak level at 1.0 μ g/ml (Fig. 2B). In

the time-dependent assay, the induction of luciferase activity in IL-4/Luc/CNS-1 Tg mice was increased significantly depending on the time of culturing with ovalbumin, reaching a peak at 72 h, whereas it was unaltered in non-Tg mice (Fig. 2A). According to these results, the optimal condition for the detection of luciferase activity in response to ovalbumin was determined to be 1.0 μ g/ml for 72 h. Furthermore, among the five Tg lines, a high level of luciferase activity was noted in the splenocytes of #57 IL-4/Luc/CNS-1 Tg mice relative to the non-Tg mice (Fig. 2C). The #57 line of the IL-4/Luc/CNS-1 Tg mice was selected for further study.

Verification of No Difference between IL-4/Luc/CNS-1 Tg Mice and Non-Tg Mice in Other Allergic Responses to PA except IL-4 Response

First, in order to determine whether or not the same allergenic response in IL-4/Luc/CNS-1 Tg mice as in non-Tg mice can be induced, with the exception of a luciferase

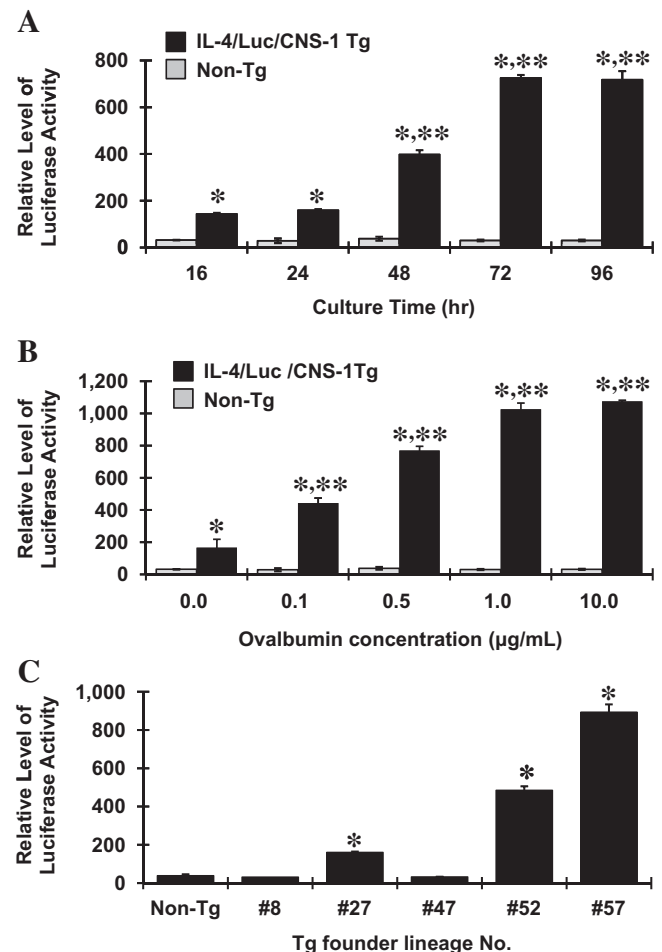


FIG. 2. Change in luciferase activity after ovalbumin treatment. Splenic cells (1×10^6) from IL-4/Luc/CNS-1 Tg incubated with ovalbumin, and the activity level of luciferase was detected in time- (A) and dose (B)-dependent patterns. (C) Luciferase activity was measured in five Tg lineages under optimal ovalbumin treatment conditions using splenocytes.

response under the IL-4 promoter, changes in various immune-related factors such as ear thickness, IgE concentration, the number of inflammatory leukocytes that infiltrated inflamed tissues, and MPO activity were detected in IL-4/Luc/CNS-1 Tg mice and non-Tg mice after PA treatment. The results of ear thickness analysis revealed that PA treatment induced an increase in ear thickness, whereas AOO treatment induced no changes. However, no differences in ear thickness were detected between IL-4/Luc/CNS-1 Tg mice and non-Tg mice.

With regard to serum IgE concentration, IL-4/Luc/CNS-1 Tg mice and non-Tg mice evidenced similar patterns in the PA treatment and AOO treatment groups, although the BALB/c mice evidenced higher IgE concentration than was seen in the Tg and non-Tg mice that originated from the C57BL/6 mice (Fig. 3B).

Furthermore, alterations in other immune-related factors, including epidermal thickness and the number of infiltrating inflammatory leukocytes, were not detected between the IL-4/Luc Tg and non-Tg mice. As demonstrated in Figures 3C, 3D, and 3E, the PA treatment induced an increase in epidermal thickness and the number of inflammatory leukocytes and MPO activity, as compared with vehicle treatment. However, the rate of increase in IL-4/Luc/CNS-1 Tg mice was very similar to that observed in non-Tg mice, whereas it was reduced in the BALB/c mice. Taken together, all the above results reveal that the IL-4/Luc/CNS-1 Tg and non-Tg mice employed in this study evidenced the same responses to PA treatment in regard to the factors of immune-related indicators besides the IL-4 luciferase response.

Induction of IL-4 Promoter-Driven Luciferase Expression by Respiratory Sensitizer Treatment in IL-4/Luc/CNS-1 Tg Mice

To investigate whether treatment with respiratory sensitizers can induce luciferase expression in various organs of IL-4/Luc/CNS-1 Tg mice, the luciferase signal from each organ was quantified using LivingImage software after individual PA or TMA treatments. The luciferase signal was detected only in the IL-4/Luc/CNS-1 Tg mice treated with PA or TMA, and their signal pattern was basically quite similar in both treatment groups (Figs. 4A and 4B). Additionally, this signal increased with increasing concentrations of PA or TMA (data not shown). Among eight organs in IL-4/Luc/CNS-1 Tg mice treated with 5% PA or 10% TMA, the highest luciferase signals were observed in the mesenteric lymph node, followed by the thymus and lung. However, AOO treatment induced no alterations in the luciferase signals observed in eight organs of IL-4/Luc/CNS-1 Tg mice (Figs. 4A, 4B, and 4C). Furthermore, similar results were noted in the luciferase activity assay using a luminometer (Fig. 4C).

Additionally, in order to verify whether the luciferase signal originated from the IL-4/Luc/CNS-1 gene was consistent with the expression of cytokines originated from endogenous genes

in Tg mice, the expression levels of four cytokines were detected in the total mRNA of various organs after PA treatment, and their results were compared with the luciferase activity. Generally, in allergic individuals, exposure to allergens results in the activation of antigen-specific Th2 cells and the secretion of a variety of cytokines and inflammatory mediators that provoke the inflammatory response (Cousins *et al.*, 2008). Among the numerous cytokines secreted by activated Th2 cells, four major cytokines including IL-4, IL-5, IL-13, and TNF- α are responsible for many of the features of allergic inflammation. As shown in Figure 4D, the mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) and proinflammatory cytokines (TNF- α) were increased markedly in the lung, mesenteric lymph node, and thymus. However, AOO treatment induced lower expressions of IL-4, IL-5, IL-13, and TNF- α in the same tissues of IL-4/Luc/CNS-1 Tg mice. Therefore, these results indicate that the expression patterns of cytokines in each organ of IL-4/Luc/CNS-1 Tg mice were exactly identical to the signal pattern of luciferase activity. Taken together, our results demonstrated that the IL-4 promoter of the IL-4/Luc/CNS-1 gene in Tg mice may respond successfully to respiratory allergens such as PA and TMA.

Induction of IL-4 Promoter-Driven Luciferase Expression by Vaccine Additive Treatment in IL-4/Luc/CNS-1 Tg Mice

In order to investigate whether treatment with vaccine additives can induce luciferase expression in various organs of IL-4/Luc/CNS-1 Tg mice, the luciferase signal from each organ was quantified using LivingImage software after ovalbumin or gelatin treatment. In the saline-injected group, no luciferase signals were detected in any organs of the IL-4/Luc/CNS-1 Tg mice. However, the highest luciferase signal was noted in the mesenteric lymph node, followed by the submandibular lymph node and thymus of IL-4/Luc/CNS-1 Tg mice treated individually with ovalbumin or gelatin. Generally, the signal pattern of luciferase was very similar in each organ of the IL-4/Luc/CNS-1 Tg mice groups treated with two different vaccine additives, although the levels observed in the ovalbumin treatment group were higher than in the gelatin treatment group. Among eight organs of IL-4/Luc/CNS-1 Tg mice treated with ovalbumin, the mesenteric lymph node, thymus, and submandibular lymph node evidenced increased luciferase signal levels (Figs. 5A and 5C). Additionally, in the luciferase activity assay using a luminometer, high levels of activity were detected only in these three organs (Fig. 5C). In order to verify the expression pattern relationship between the luciferase signal originating from the IL-4/Luc/CNS-1 gene and the cytokines originating from the endogenous gene, the level of four cytokines were detected in the total mRNA of various organs after ovalbumin treatment. All Th2 cytokines (IL-4, IL-5, and IL-13) were highly expressed in only the organs in which a luciferase signal was detected, although the high-level induction of a proinflammatory cytokine (TNF- α) was noted

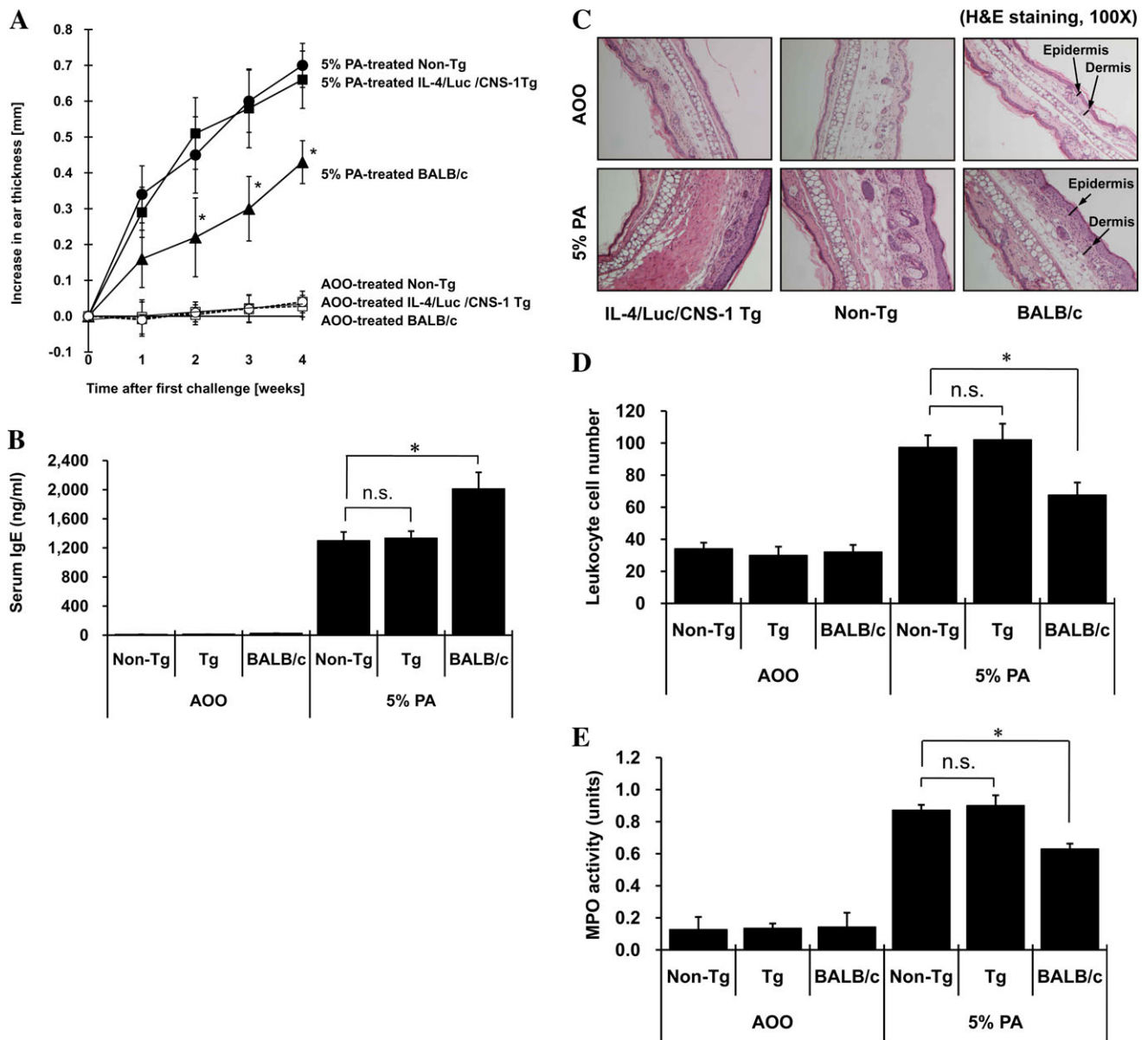


FIG. 3. Induction of the same allergenic response to PA in both IL-4/Luc/CNS-1 Tg mice and non-Tg mice. Changes in various immune-related factors such as ear thickness (A), IgE concentration (B), increases in epidermal thickness (C), the number of inflammatory leukocytes infiltrated into inflamed tissue (D), and MPO activity (E) were detected.

only in the mesenteric lymph node. However, saline treatment induced low-level IL-4, IL-5, IL-13, and TNF- α expression in the same tissues of the IL-4/Luc/CNS-1 Tg mice (Fig. 5D).

IgE hyperproduction is one of the characteristic features of allergic hypersensitivity and functions as an indicator of the magnitude of allergic immune response to an allergen (Dearman *et al.*, 2003). Ovalbumin as an allergen induced IgE hyperproduction in IL-4/Luc/CNS-1 Tg mice and non-Tg mice. However, the IL-4/Luc/CNS-1 Tg mice and non-Tg mice evidenced similar patterns in both the saline treatment and the ovalbumin treatment group (Fig. 5E). Taken together, these results indicate that IL-4/Luc/CNS-1 Tg mice may be

employed in the evaluation of risk for vaccine additives including ovalbumin and gelatin.

Induction of IL-4 Promoter-Driven Luciferase Expression by Treatment with a Crude Extract of Natural Allergen in IL-4/Luc/CNS-1 Tg Mice

In order to determine whether a crude extract of natural allergen treatment can induce luciferase expression in various organs of IL-4/Luc/CNS-1 Tg mice, the luciferase signal from each organ after DPE, JCP, and CE treatment was quantified with LivingImage software. As can be observed in Figures 6A, 6B, and 6C, DPE and JCP treatment induced luciferase signals

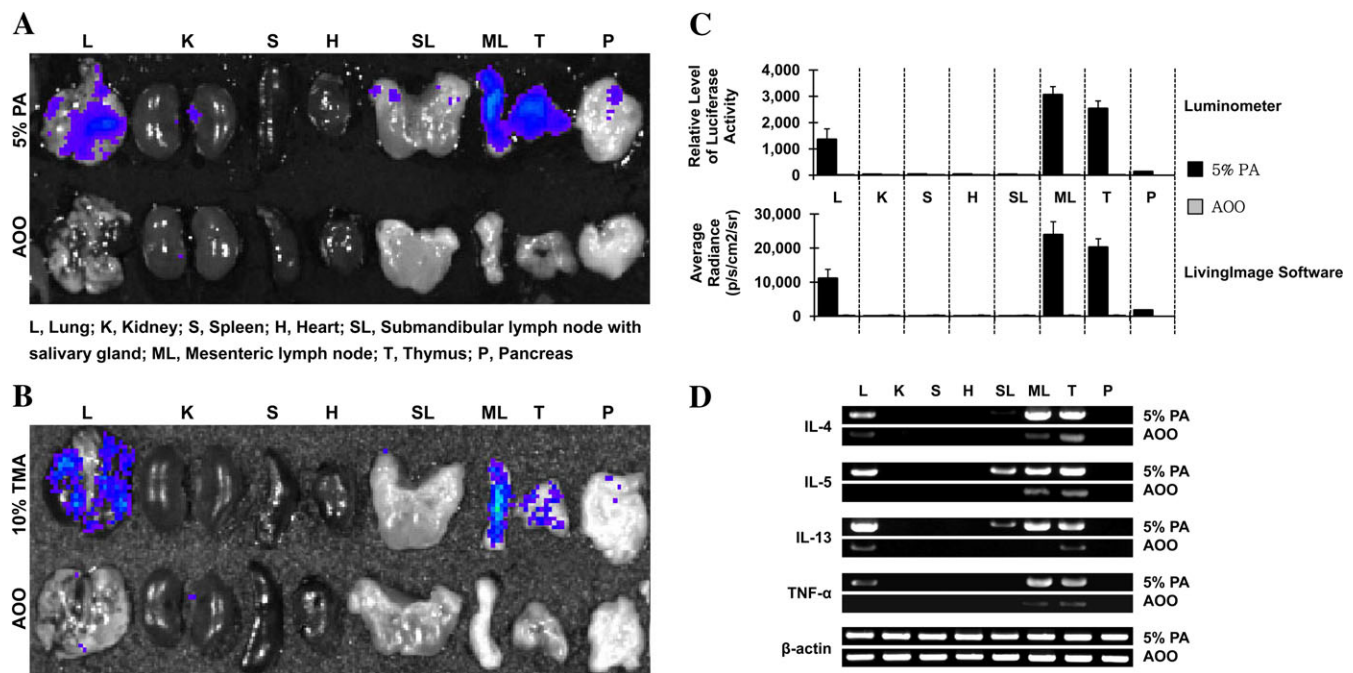


FIG. 4. Induction of IL-4 promoter-driven luciferase expression by respiratory sensitizer treatment. Isolated organs after PA (A) and TMA (B) treatment were imaged with LivingImage software. (C) Bioluminescent signals shown by LivingImage software (upper panel) were compared with the luciferase activity of the tissue extract in the AOO- and PA-treated group using a luminometer (low panel). (D) Expressions of cytokines in various tissues were detected in the AOO- and PA-treated groups via RT-PCR analysis.

only in the lung, whereas no alterations were detected in other organs. However, CE treatment induced no luciferase signals in any organs of the IL-4/Luc/CNS-1 Tg mice. Additionally, in the AOO treatment group, needless to say, no luciferase signals were detected in any organs of IL-4/Luc/CNS-1 Tg mice. In the luciferase activity assay using a luminometer, activity was detected only in the lungs of DPE-treated IL-4/Luc/CNS-1 Tg mice (Fig. 6D). In particular, lung inflammation was visible with the naked eye only in the DPE treatment group (Fig. 6G). Additionally, cytokine expression analysis showed that the mRNA expression of Th2 cytokines and proinflammatory cytokine were markedly increased only in the lung. However, the nontreated group of IL-4/Luc/CNS-1 Tg mice exhibited no high-level inductions of IL-4, IL-5, IL-13, and TNF- α in any organs (Fig. 6E). Furthermore, DPE did not induce IgE hyperproduction in IL-4/Luc/CNS-1 Tg mice and non-Tg mice (Fig. 6F). Therefore, these results indicate that IL-4/Luc/CNS-1 Tg mice may be employed in quantitative evaluations of the risk of crude extracts of natural allergens such as DPE and JCP.

DISCUSSION

Well-known allergens including respiratory sensitizer, vaccine additives, and crude extracts of natural allergens were selected for application to IL-4/Luc/CNS-1 Tg mice. Among respiratory sensitizers, PA is an important industrial chemical, particularly in the large-scale production of plasticizers for

plastics (Hayashi *et al.*, 2001). In the human body, the eye is irritated by PA, which occurs as a vapor, a fume, or a dust. The skin and respiratory tract are also sensitized and irritated by PA treatment. PA treatment may also generate asthma upon repeated exposure (Ban and Hettich, 2005; Dearman and Kimber, 1992). TMA is used in the manufacture of plasticizers, resins, polymers, dyes, and printing inks. Additionally, TMA is a representative example of a chemical sensitizer associated with occupational asthma and allergic rhinitis in industrial settings (Farraj *et al.*, 2004). In the previous studies, mice that were challenged with TMA evidenced a marked allergic rhinitis with an influx of eosinophils, lymphocytes, and plasma cells; increased intraepithelial mucus substances; regenerative hyperplasia; and an increase in the mRNA of the Th2 cytokines IL-4, IL-5, and IL-13 (Farraj *et al.*, 2006). This study demonstrated that the mesenteric lymph node located between the layers of the mesentery evidenced the most profound response to PA or TMA. In this organ, T cells expressed higher levels of IL-4 and IL-5 mRNA than were seen in the circulating lymphocytes. Furthermore, T cells in the mesenteric lymph node can proliferate in response to IL-4 (James, 1991). Therefore, the highest levels of luciferase activity in the mesenteric lymph node may be considered one relevant characteristic of T cells.

In the lung, increases in the levels of IgE and Th2 cytokines are associated with the promotion of airway hyperresponsiveness, mucus secretion, and increased vascular permeability (Passalacqua and Ciprandi, 2008). Furthermore, several studies

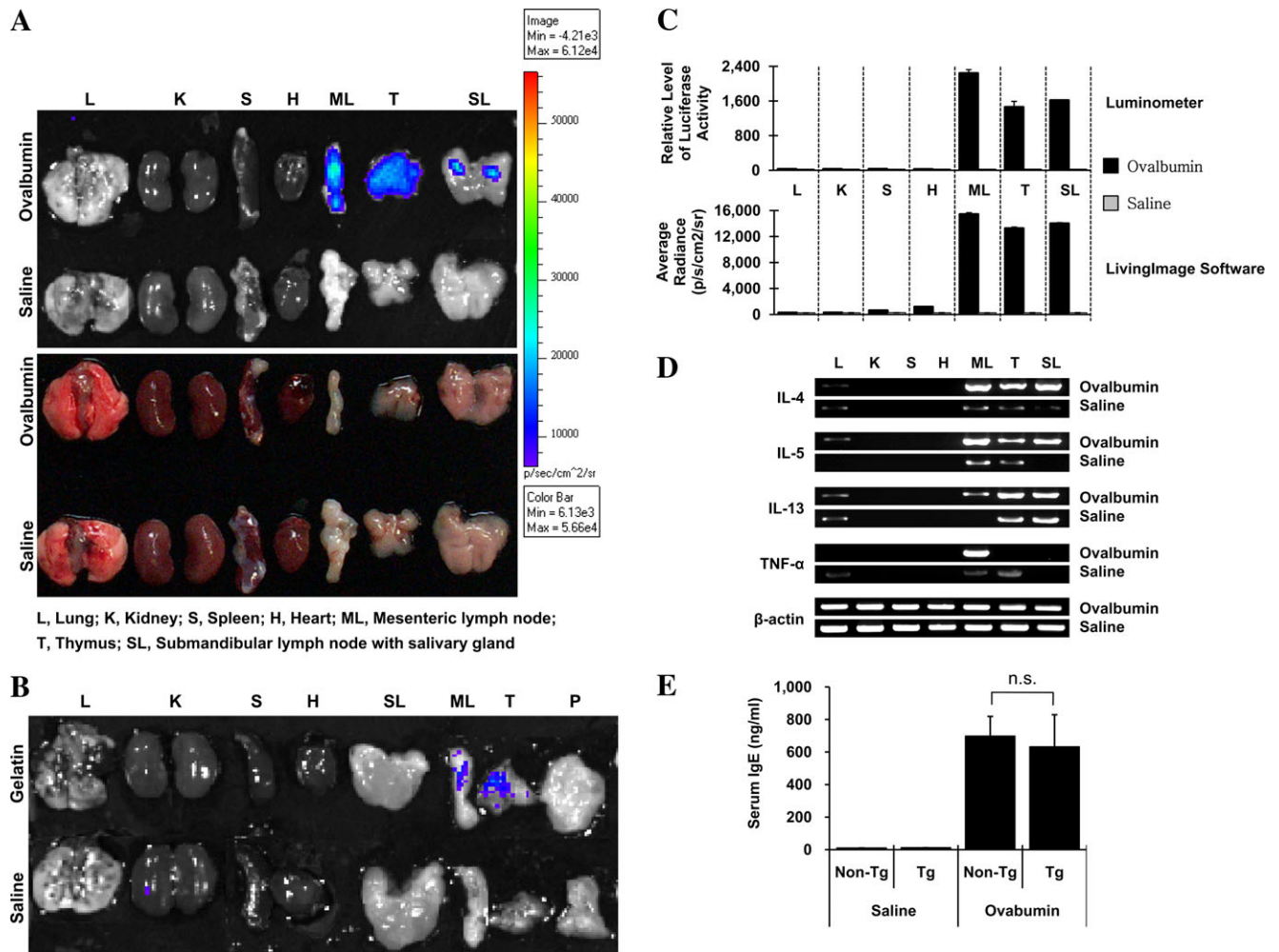


FIG. 5. Induction of IL-4 promoter-driven luciferase expression by vaccine additive treatment. Isolated organs after ovalbumin (A) and gelatin (B) treatment were imaged with LivingImage software. (C) Bioluminescent signals from LivingImage software (upper panel) were compared with the luciferase activity of the tissue extract in the saline- and ovalbumin-treated groups using a luminometer (low panel). The expressions of cytokines in various tissues (D) and concentrations of serum IgE (E) were detected in the saline- and ovalbumin-treated groups.

involving IL-4-deficient and IL-13-deficient mice have demonstrated that IL-4 and IL-13 are the cytokines that principally contribute to airway hyperresponsiveness and inflammation (Brusselle *et al.*, 1994; Walter *et al.*, 2001). Also, the thymus, a specialized immune system organ composed of numerous lobules conjugated together by delicate areolar tissue, provides an excellent environment for T lymphocyte development from hematopoietic progenitor cells (Miller, 2002). Many T cells belonging to the thymus may perform an important function in high luciferase activity following PA or TMA treatment. Overall, based on these results, the lung and thymus of IL-4/Luc/CNS-1 Tg mice evidenced a profound response to PA or TMA treatment. Taken together, our results reveal that IL-4/Luc/CNS-1 Tg mice constructed on the basis of an IL-4 responsible element were quite suitable for the detection of respiratory sensitizers such as PA and TMA.

On the one hand, we selected two vaccine additives including ovalbumin and gelatin to determine whether luciferase expression in various organs of IL-4/Luc/CNS-1 Tg mice could be induced by vaccine additive treatments. Among vaccine additives, ovalbumin is the main protein found in egg white, comprising 60–65% of the total protein, and is also an important protein in various other areas of research, including protein structure, proteomics, and immunology (Huntington and Stein, 2001). In the manufacturing process for most vaccines, varying quantities of residual ovalbumin and gelatin are added to stabilize the protein. However, ovalbumin appears to induce severe, and rarely fatal, hypersensitivity reactions in children with egg allergies (James *et al.*, 1998). It is therefore important to evaluate the amount of ovalbumin and other impurities in the vaccine (Kursteiner *et al.*, 2006). Also, gelatin is usually included in live-virus vaccines such as measles, mumps, rubella, and varicella vaccines. Most of the

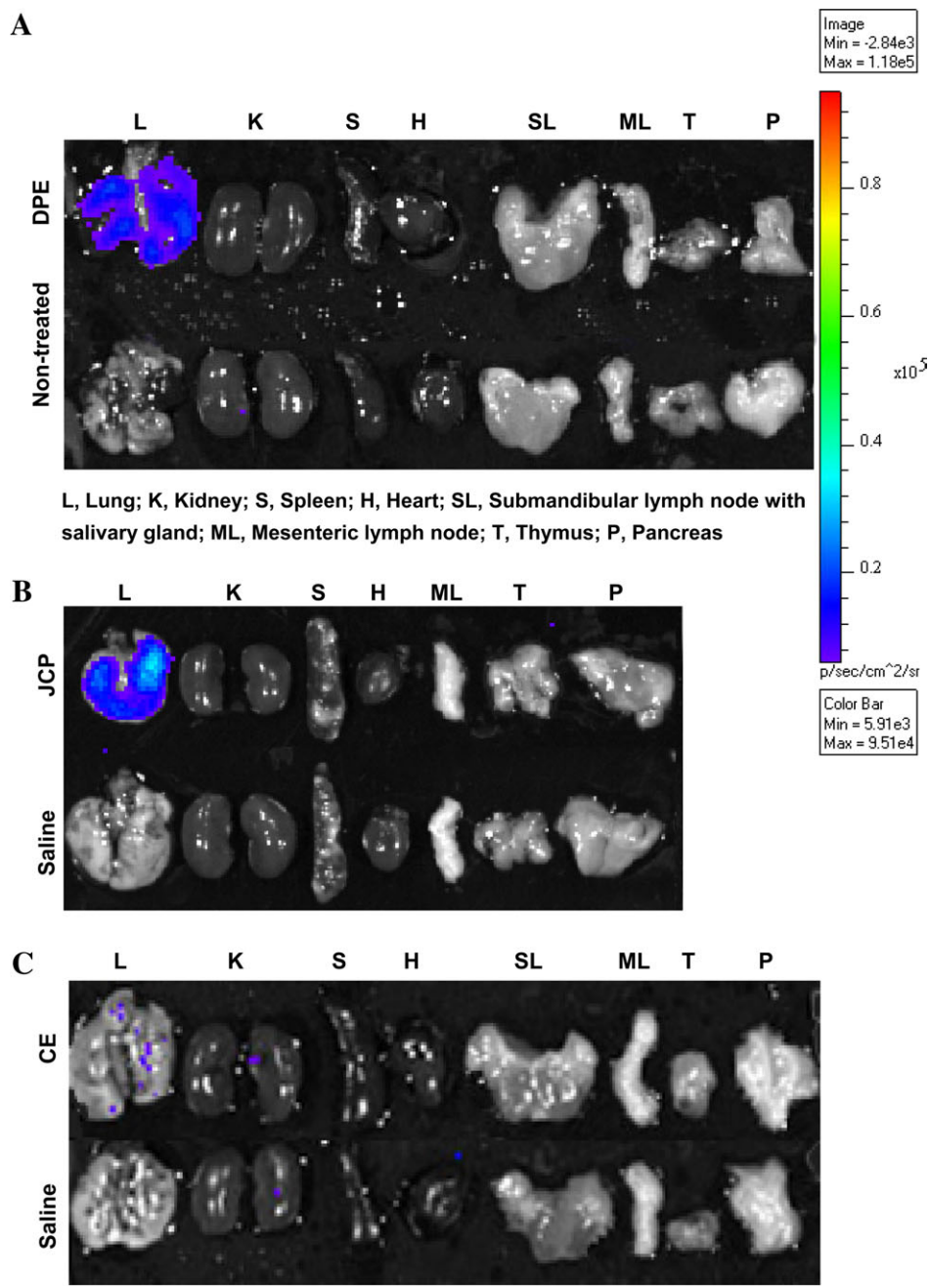


FIG. 6. Induction of IL-4 promoter-driven luciferase expression by treatment with crude extracts of natural allergens. Isolated organs after DPE (A), JCP (B), and CE (C) treatments were imaged with LivingImage software. (D) Bioluminescent signals from LivingImage software were compared with the luciferase activity of the tissue extract in the nontreated and DPE-treated groups using a luminometer. Cytokines in various tissues (E) and IgE in serum (F) were detected in the nontreated and DPE-treated groups. (G) Inflamed lungs in the nontreated and DPE-treated groups are visible with LivingImage software and with the naked eye.

children evidencing systemic immediate-type reactions, including anaphylactic shock, evidenced IgE antibody responses to gelatin (Kuno-Sakai and Kimura, 2003). In this study, the expression patterns of the luciferase signal in each organ were even maintained in IL-4/Luc/CNS-1 Tg mice treated with two different vaccine additives, although the ratio of expression level was different. Therefore, these results demonstrated that

IL-4/Luc/CNS-1 Tg mice may be employed effectively for the detection of various vaccine additives. Furthermore, in this study, we applied different methods for the treatment of animals with respiratory sensitizers and vaccine additives. Respiratory sensitizer was repeatedly spread on each ear and the shaved dorsal region of mice, whereas the vaccine additives were administered via ip injection. However, similar luciferase

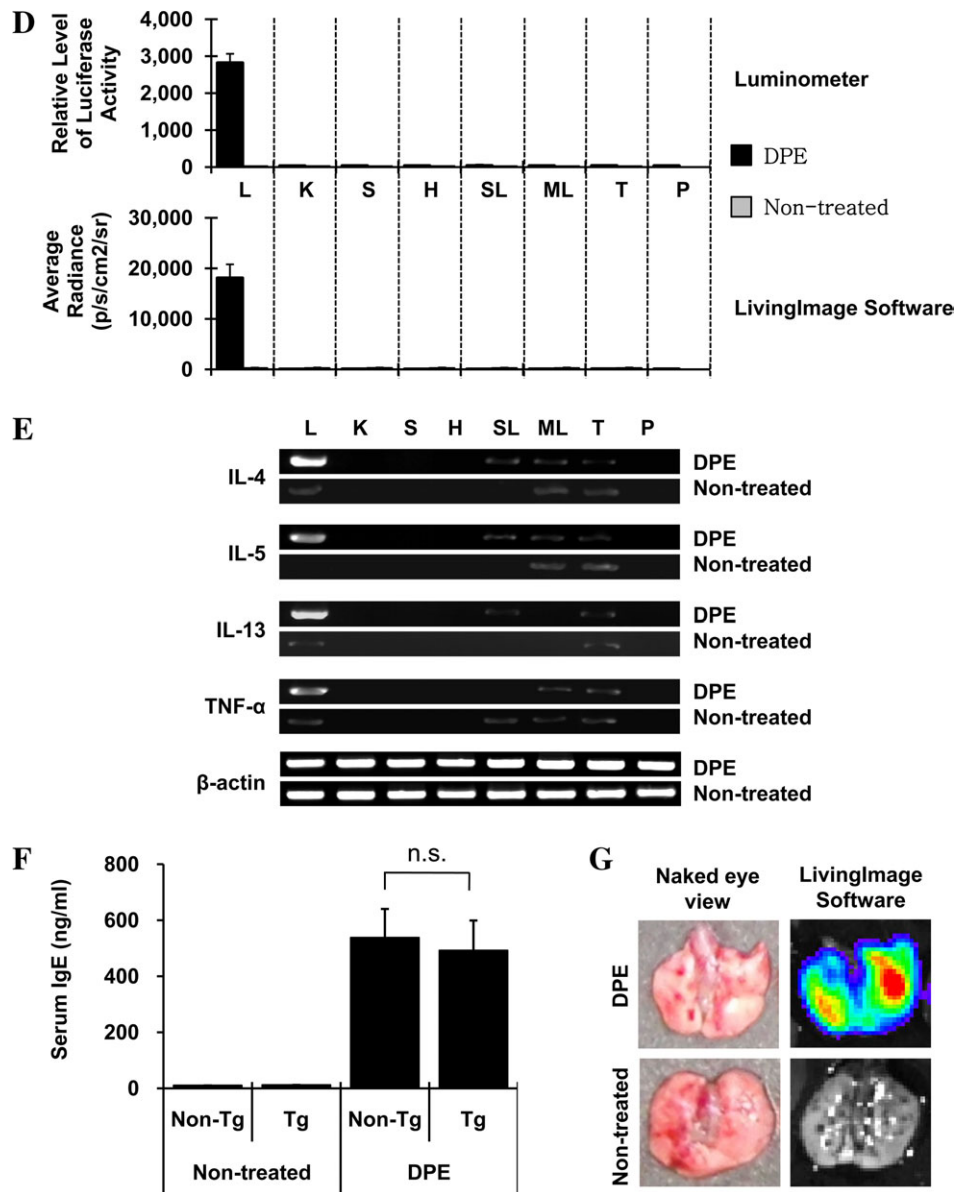


FIG. 6. Continued.

signals were detected in various organs, except the lung. Therefore, our results indicate that the IL-4 response may be determined more by allergen characteristics than by routes of administration.

Finally, we attempted to determine whether IL-4/Luc/CNS-1 Tg mice could respond to crude extracts of natural allergens such as DPE, JCP, and CE. Among these natural allergens, *D. pteronyssinus* contains important allergic proteins involving Der p1 and Der p2. House dust mites also induce the expression of Th2 cytokines including IL-4, IL-5, and IL-13 in animal bronchoalveolar lavage and lungs (Gregory *et al.*, 2009). Furthermore, Tg mice expressing the T cell antigen receptor for an immunodominant epitope of a major house dust

mite allergen were generated in an effort to develop an *in vivo* model of allergic asthma (Jarman *et al.*, 2005). These Tg mice evidenced asthmatic phenotypes, including the functional activation of CD4⁺ T cells, IL-4 production, and airway inflammation. Additionally, JCP was reported as a causative allergen of pollinosis, a type of seasonal allergic rhinitis and the most common allergic respiratory disease. Repeated intranasal sensitization with Cry j1, the major allergen of JCP, in the absence of adjuvants significantly exacerbated nasal hyperresponsive symptoms and high levels of IL-4 and IL-5 in the nasal mucosa (Nomiya *et al.*, 2008). In our study, DPE induced a high response only in the lungs of IL-4/Luc/CNS-1 Tg mice, whereas it induced no luciferase signal in any other organs.

Therefore, these results also reinforce that the lung is a major target organ showing an asthmatic phenotype after DPE treatment. Taken together, our study demonstrated that IL-4/Luc/CNS-1 Tg mice may be applied to the evaluation of house dust mites and pollen.

As is shown in Figures 4D, 5D, and 6E, the cytokine profiling method may also be used for the evaluation and prediction of the allergenicity of unknown materials. However, the bioluminescence imaging method using Tg mice may prove an easier or more practical allergen detection method, as compared with cytokine profiling. For cytokine profiling, total RNA should be isolated from a number of organs. Additionally, reverse transcription and PCR reaction should be conducted. These steps are laborious and time consuming. However, in the bioluminescence imaging system, cytokine expression levels from various organs can be evaluated only via ip injections of D-luciferin as a substrate for luciferase. Moreover, living mice can be employed in a bioluminescence imaging system. This method is beneficial in that it requires a reduced number of mice and can detect luciferase activity in real time.

In conclusion, all the aforementioned results demonstrate that IL-4/Luc/CNS-1 Tg mice can function as an animal model for the evaluation and prediction of human body responses against a variety of environmental allergens and for the study of the mechanisms of action and efficacy of novel immunotherapeutic agents.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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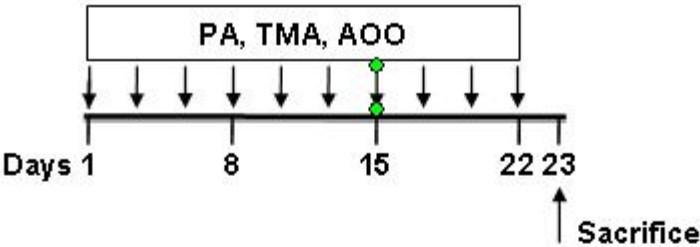
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Supplemental table 1. List of allergen treated in IL-4/Luc/CNS-1 Tg mice

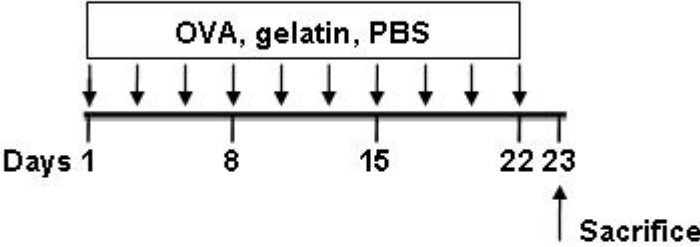
Allergens			Organs expressing the luciferase
Kinds	Administration		
	Dose	Route	
Respiratory sensitizer			
Phthalic anhydride	5 mg	Skin	L, ML, T
Trimellitic anhydride	10 mg	Skin	L, ML, T
Vaccine additives			
Ovalbumin	50 µg	i.p.	ML, T, SL
Gelatin	50 µg	i.p.	ML, T
Protein			
<i>D. pteronyssinus</i> crude extract	100 mg	Skin	L
Japanese cedar pollen	5 µg	Intranasal	L
Cockroach extract	5 µg	Intranasal	n.d.
L, Lung; K, Kidney; S, Spleen; H, Heart; SL, Submandibular lymph node with salivary gland; ML, Mesenteric lymph node; T, Thymus; P, Pancreas; n.d, non-detected			

Supplemental figure 1

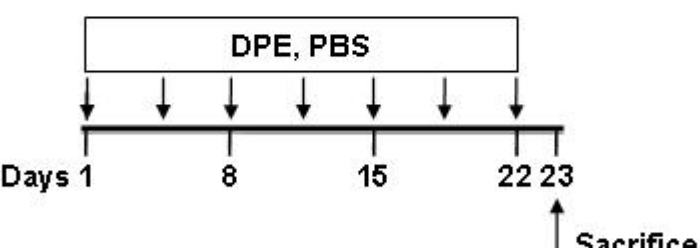
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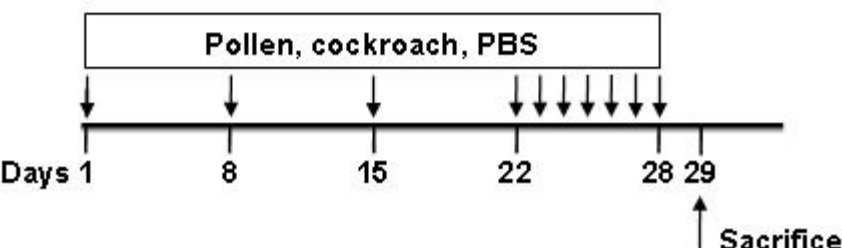
B



C



D



1 Supplemental Figure Legends

2

3 **Supplemental figure 1. Time schedules for treating allergens.** (A) Either chemical
4 sensitizers such as PA and TMA (Trimellitic anhydride) or AOO were repeatedly spread on
5 the shaved dorsal region of each mice 3 times a week for 3 weeks. (B) Either vaccine
6 additives including OVA and gelatin or PBS were injected i.p. 3 times a week for 3 week.
7 (C) DPE was applied on the shaved dorsal skin twice a week for 3 weeks. (D) Tg mice (5
8 per group) were sensitized by intranasal administration of either 5 μ g of Japanese cedar
9 pollen or cockroach extract in 10 μ l PBS in the absence of adjuvants once a week for 3
10 weeks (on days 1, 8, and 15). One week after the third sensitization, mice were challenged
11 by intranasal administration every day for 7 consecutive days (on days 22 to 27). As control,
12 mice were treated with PBS.