

Color-coded real-time cellular imaging of lung T-lymphocyte accumulation and focus formation in a mouse asthma model

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Background: A critical role for CD4⁺T_H2 cells in the pathogenesis of acute asthma has been demonstrated in the studies of human asthma as well as of animal models of asthma. T_H2-cell migration into the lung is crucial for the initiation of asthma phenotype, but the dynamics of this process are poorly understood because it has been difficult to visualize this process. **Objective:** Our aim was to image the cellular dynamics of the migration of T_H2 cells into the lung of living animals in a mouse model of asthma and identify the cellular processes required for the initiation of the asthma phenotype.

Methods: We developed a color-coded real-time imaging model of cell migration into the lung using green fluorescent protein (GFP) and red fluorescent protein (RFP) transgenic CD4 T cells.

Results: Selective accumulation of antigen-specific CD4 T cells in the lungs was quantitatively imaged in a mouse model of asthma. The inhibition of accumulation by dexamethasone was imaged. Accumulating GFP⁺ T_H2 cells formed foci in the lungs from 6 to 20 hours after antigen inhalation. This process was also inhibited by the administration of anti-intercellular adhesion molecule 1 or anti-vascular cell adhesion molecule 1 mAbs. Two days after inhalation of antigen, GFP⁺ T_H2 cells were detected in the area of eosinophil infiltration.

Conclusion: Focus formation generated by accumulating antigen-specific T_H2 cells in the lung appeared to be a critical process in the initiation of the asthma phenotype. This new model enables the study of *in vivo* cell biology of airway inflammation and novel drug discovery for lung inflammatory diseases. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Real-time *in vivo* cellular imaging, cellular dynamics, T_H2 cells, mouse model of asthma, focus formation, airway inflammation, GFP, ICAM-1, VCAM-1

Lung inflammatory diseases such as asthma are major public health problems that have increased markedly in prevalence in the past 3 decades.¹ Asthma is characterized by a chronic inflammatory disease of the lower airways that causes airway hyperresponsiveness to a wide variety of specific and nonspecific stimuli.^{2,3} The cardinal features of acute asthma are airway inflammation predominated by eosinophils, hypersecretion of mucus, and airway hyperresponsiveness. A critical role for CD4⁺ T_H2 cells in the pathogenesis of acute asthma has been demonstrated in studies of human asthma as well as in animal models of allergic airway inflammation.⁴⁻¹⁰ Previous investigations have studied T-cell migration during allergic reactions by using an adoptive cell transfer system.¹¹⁻¹³ The administration of anti-intercellular adhesion molecule 1 (ICAM-1) or anti-vascular cell adhesion molecule 1 (VCAM-1) antibody resulted in the inhibition of eosinophilic airway inflammation.¹⁴ However, the *in vivo* dynamics of cell migration into the lung during inflammation in living animals is poorly understood because of the lack of an appropriate *in vivo* cellular imaging model. The use of fluorescent proteins for imaging, which we pioneered, allowed us to monitor cell migration *in vivo*.¹⁵ Green fluorescent protein (GFP) and red fluorescent protein (RFP) were used to label living cells genetically *in vivo* as well as *in vitro*^{16,17} and served as a powerful tool to monitor the migration of specific lymphocytes in live animals.

We report here a novel *in vivo* real-time color-coded cellular imaging model to visualize the dynamics of migration of T cells in a mouse model of asthma. We have found that accumulating T_H2 cells formed foci in the lungs 6 to 20 hours after allergen inhalation. Focus formation was dependent on ICAM-1 and VCAM-1 and appeared to determine the site of eosinophilic infiltration, indicating that T_H2-cell focus formation is a critical process during the initiation of airway inflammation in this animal model.

METHODS

Mice

C57BL/6 were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6-transgenic (Tg) (CAG-EGFP)C14-Y01-FM1310sb (GFP Tg, C57BL/6 background) mice expressed an enhanced GFP in all tissue under the control of the β -actin promoter.¹⁶ C57BL/6 background RFP Tg mice expressed RFP (DsRed-2) under the control of the chicken β -actin promoter and cytomegalovirus enhancer.^{17,18} Ovalbumin-specific T-cell receptor $\alpha\beta$

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Abbreviations used

DEX:	Dexamethasone
GFP:	Green fluorescent protein
H&E:	Hematoxylin and eosin
ICAM-1:	Intercellular adhesion molecule 1
NIH:	National Institutes of Health
RFP:	Red fluorescent protein
TCR:	T-cell receptor
Tg:	Transgenic
VCAM-1:	Vascular cell adhesion molecule 1

transgenic (OT II Tg) mice¹⁹ were maintained under specific pathogen-free conditions. All animal care was carried out in accordance with the National Institutes of Health (NIH) guidelines (NIH assurance no. A3873-01) at AntiCancer, Inc, and the guidelines of Chiba University. All animal experiments were performed at AntiCancer Inc.

In vitro T_H2-cell differentiation cultures

Green fluorescent protein Tg x OT II Tg CD44^{low} CD4 T cells (2×10^5) were purified by cell sorting and stimulated with antigenic ovalbumin peptide (Loh 15, 1 μ M/L) and irradiated (30 Gy) syngeneic antigen-presenting cells (1×10^6) in the presence of exogenous IL-4 as described previously.²⁰

Ovalbumin sensitization, cell transfer, and ovalbumin inhalation

The GFP or RFP Tg mice were immunized intraperitoneally with 250 μ g ovalbumin (chicken egg albumin from Sigma St Louis, Mo) in 4 mg aluminum hydroxide gel (alum) on days 0 and 7. Splenic CD4 T cells from ovalbumin-sensitized GFP or RFP Tg mice were isolated by magnetic negative selection using a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany) on day 14, yielding a purity of >98%. These cells (2×10^7 cells) or ovalbumin-specific T_H2 cells (5×10^6 cells) were transferred intravenously through the tail vein to 8-week-old C57BL/6 recipient mice. One or 2 days later, the recipient mice inhaled aerosolized ovalbumin in saline (10 mg/mL) for 30 minutes with a supersonic nebulizer (NE-U07; Omron Co, Kyoto, Japan) as described previously.²¹

Fluorescence imaging of cell accumulation in the lung

The mice were killed by CO₂ asphyxiation at various times after ovalbumin inhalation. The lungs were removed, and GFP⁺ cells that had accumulated in the excised lung were monitored by using an OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan).^{15,22,23}

Dexamethasone treatment

Splenic CD4 T cells (2×10^7 cells) were transferred intravenously through the tail vein to 8-week-old C57BL/6 recipient mice on day 14. One hour before the airway challenge by ovalbumin inhalation (on day 15), mice were injected intraperitoneally with dexamethasone (0.4, 1, or 4 mg/kg). The mice were exposed to allergen challenges on days 15, and transferred GFP⁺ CD4 T cells were monitored on day 16. Where indicated, dexamethasone was injected 1 hour before (day 15) or 1 day after ovalbumin inhalation (day 16). Two days after OVA inhalation (day 17), GFP⁺ CD4 T cells were monitored by using the OV100 Small Animal Imaging System.

Anti-ICAM-1 and anti-VCAM-1 antibody treatment

Ovalbumin-specific T_H2 cells (5×10^6 cells) were transferred intravenously through the tail vein of 8-week-old C57BL/6 recipient mice. Twenty-four hours before the airway challenge by ovalbumin inhalation,

mice were injected intraperitoneally with 200 μ g anti-ICAM-1 (YN1/1.7.4) or anti-VCAM-1 (429) mAbs.¹⁴ One day after ovalbumin inhalation, transferred ovalbumin-specific T_H2 cells were monitored by using the OV100 Small Animal Imaging System.

In vivo imaging of lung infiltrating T cells by scanning laser microscopy

Control mice and mice given ovalbumin were prepared surgically at various indicated time points after ovalbumin administration for lung imaging. The mice were anesthetized and tracheostomized on the surgical bed and kept at 37°C. The right bronchus was clamped to stop movement during ventilation. The left lung was mechanically ventilated with O₂ at the normal respiratory rate to keep the mice alive. The clamped right lung was monitored with the IV100 scanning laser microscope (Olympus Corp., Tokyo, Japan). The IV100 microscope enabled imaging up to 100 μ m depth from the surface of the lung. A 488-nm argon laser was used. To create an *in vivo* video image, images were recorded at 5-second intervals for 40 minutes. A focus was scored when more than 50% of the 2-dimensional area was occupied by the infiltrating GFP⁺ cells. Crawling (motile) cells in the lung were defined as those that migrated or elongated to more than 50% of their diameter. The NIH Image software program (NIH Image J 1.41) was used for image analysis.

Lung histology and immunohistochemistry

The mice were killed by CO₂ asphyxiation at the indicated times after ovalbumin inhalation, and the lungs were infused with 10% (vol/vol) formalin in PBS or 4% (vol/vol) paraformaldehyde for fixation. The lung samples were sectioned, stained with hematoxylin and eosin (H&E), and examined for pathological changes under a light microscope at magnification $\times 50$ or $\times 200$.⁷ Lung specimens were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Fluelek, Tokyo, Japan), frozen in liquid nitrogen, and cut with a cryostat into 6- μ m-thick sections. Endogenous peroxidase activity as well as nonspecific protein binding was sequentially blocked by using 0.6% hydrogen peroxide and Biotin-Blocking System reagent (DAKO, Glostrup, Denmark), respectively. The sections were incubated with hamster anti-GFP mAb (AdD Serotec, Oxford, UK) at 10 μ g/mL overnight at 4°C and then were washed in TRIS-buffered saline with Tween. Bound antibody was detected by sequential incubation with biotinylated rabbit antihamster IgG and streptavidin-horseradish peroxidase followed by 3,3'-diaminobenzidine (DAKO-Cyotomation). The slides were then washed and counterstained with hematoxylin.

Statistical analysis

Experimental data were expressed as the means + SDs. The significance between 2 groups was determined by the 2-tailed Student *t* test.

RESULTS**Color-coded fluorescence imaging of selective accumulation of ovalbumin-primed CD4 T cells into the lung in an ovalbumin-induced acute-asthma mouse model**

To examine the CD4 T-cell behavior in the lung of living mice in a mouse model of asthma, we developed a color-coded imaging model using GFP or RFP Tg CD4 T cells (Fig 1, A). Immediately after cell transfer, numerous transferred cells temporally and non-specifically accumulated in the lung capillaries (data not shown). One day later, some of the cells remained in the lung with no significant difference between sensitized GFP⁺ and non-sensitized RFP⁺ cells (Fig 1, B, left; before inhalation). One day after ovalbumin inhalation, the number of GFP⁺ CD4 T cells from ovalbumin-immunized mice increased significantly, and some of them

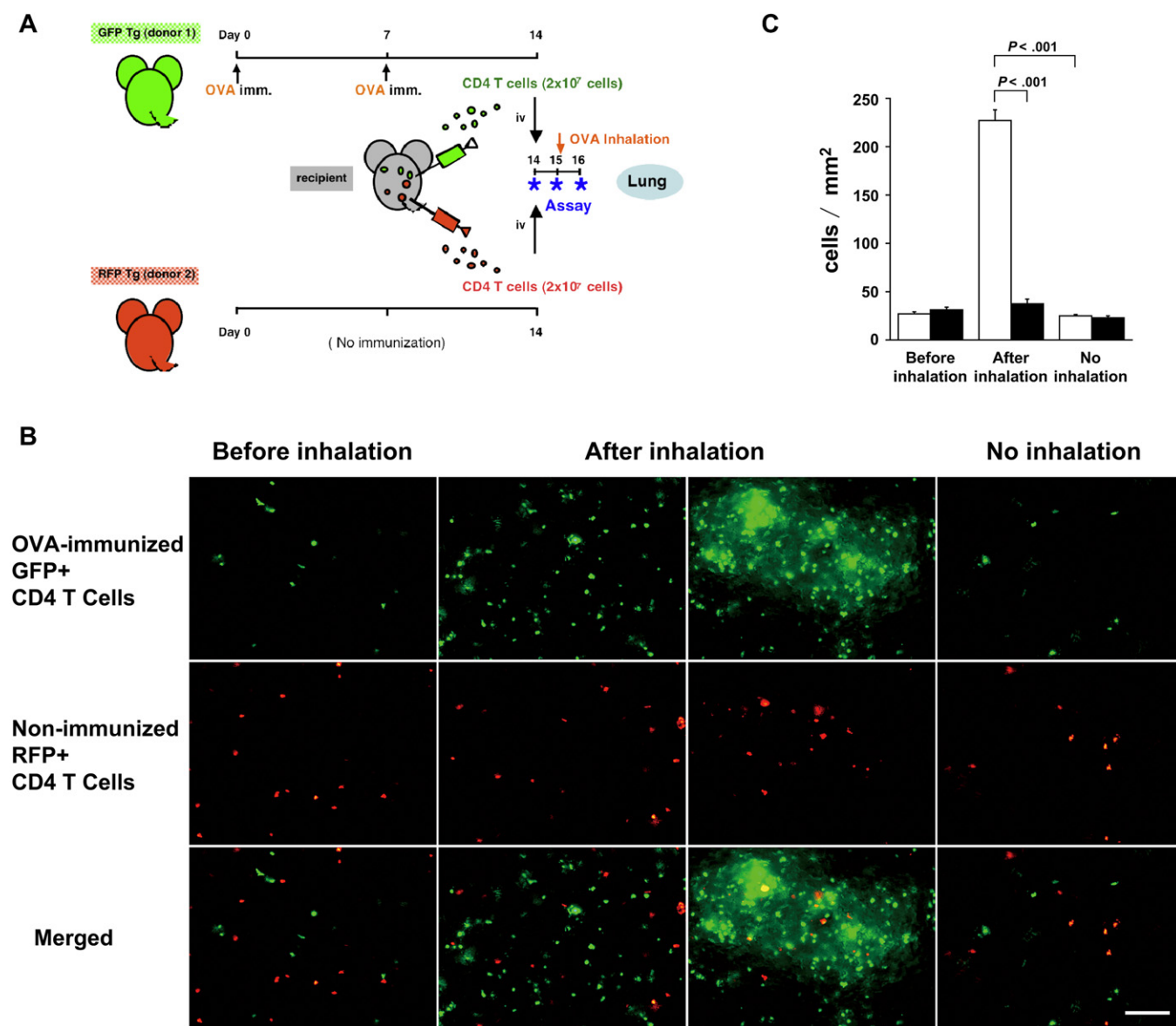


FIG 1. Color-coded fluorescence imaging of ovalbumin (OVA)-primed CD4 T-cell accumulation in the lung in the OVA-induced acute asthma model. **A**, A schematic overview of the study protocol for the induction of asthma. GFP Tg mice were sensitized with OVA on days 0 and 7. Splenic CD4 T cells from OVA-sensitized GFP Tg and nonsensitized RFP Tg mice were purified and injected into normal C57BL/6 mice on day 14. The recipient mice were exposed to airway challenge with aerosolized OVA on day 15. **B**, Color-coded images of GFP⁺ and RFP⁺ CD4 T cells. GFP⁺ and RFP⁺ CD4 T cells in the excised lung were monitored before OVA inhalation on day 15 and 24 hours after OVA inhalation on day 16 by using the OV100 Small Animal Mouse Imaging System. Bar, 100 μ m. **C**, Summary of the accumulation of fluorescent cells. Data are from 15 fields from 3 mice with SD. Open bar, GFP; closed bar, RFP. **P* < .001 by the Student's *t* test.

formed foci (Fig 1, B, center; after inhalation). In contrast, the number of RFP⁺ CD4 T cells from nonimmunized mice did not increase in the lung (Fig 1, B and C). These results indicate that CD4 T-cell migration into the lung after ovalbumin inhalation is ovalbumin priming-dependent. When we used the opposite color-coded immunization pattern, only the ovalbumin-primed RFP⁺ CD4 T cells accumulated in the lung, not the nonprimed GFP⁺ CD4 T cells (see this article's Fig E1, A and B, in the Online Repository at www.jacionline.org). These results indicate that the difference in accumulation is not fluorescent protein-dependent.

Furthermore, we examined the migration of naive antigen-specific CD4 T cells in the lung. Ovalbumin-specific CD4 T cells were prepared from nonimmunized mice derived by crossing GFP Tg mice with OT II Tg mice, which have ovalbumin-specific, MHC class II-restricted $\alpha\beta$ T-cell receptors.¹⁹ Most of the CD4 T cells in the OT II Tg mice are ovalbumin-specific. Ovalbumin-specific CD4 T cells were transferred intravenously into normal C57BL/6 mice 24 hours before airway challenge with aerosolized ovalbumin. Twenty-four hrs after ovalbumin inhalation, GFP⁺ cells in the excised lungs were monitored by fluorescence

imaging. The number of GFP⁺ OT II Tg-CD4 T cells did not increase in the lung (see this article's Fig E2 in the Online Repository at www.jacionline.org). These results indicate that even antigen-specific CD4 T cells do not accumulate in the lung after allergen challenge if the cells are not primed.

Imaging of ovalbumin-specific OT II-T_H2 cell accumulation into the lung and the generation of GFP⁺ T_H2 cell foci in a mouse model of asthma

To investigate the dynamics of accumulating antigen-specific effector T cells in the lung of living mice in a mouse model of asthma, ovalbumin-specific T_H2 cells (OT II-Tg-T_H2) were prepared *in vitro* from naive CD4 T cells obtained from GFP⁺ OT II Tg mice. Ovalbumin-specific OT II-Tg-T_H2 cells expressing GFP were transferred intravenously into normal C57BL/6 mice. Immediately after cell transfer, numerous transferred cells temporally and nonspecifically accumulated in the lung capillaries (data not shown). Twenty-four hours after cell transfer, the recipient mice were exposed to allergen challenge with aerosolized ovalbumin. GFP⁺ OT II-T_H2 cells were imaged under fluorescence microscopy at various time points as long as 24 hours after ovalbumin inhalation by excising the lung (Fig 2, A). The accumulation of GFP⁺ OT II-T_H2 cells in the lung was detected beginning 6 hours after allergen inhalation. The maximum number of OT II-T_H2 cells accumulated between 18 and 24 hours. GFP⁺ OT II-T_H2 cells formed small foci 6 hours after ovalbumin inhalation (Fig 2, A). The number of foci increased, and their mean size also increased at 12 hours. The number of foci in the lungs further increased (18 hours). Nonfocal GFP⁺ cells also greatly increased in the lung. At 18 hours or later, the border of the foci became diffuse, and many foci appeared to be fused. For color-coded imaging of the migration of nonprimed CD4 T cells around the OT II-T_H2-cell foci, GFP⁺ OT II-T_H2 cells and splenic CD4 T cells from nonsensitized RFP Tg mice were purified and intravenously transferred together into normal C57BL/6 mice 24 hours before airway challenge with aerosolized ovalbumin. Twenty-four hours after ovalbumin inhalation, GFP⁺ and RFP⁺ cells in the excised lungs were monitored by fluorescence imaging. The number of RFP⁺ CD4 T cells from nonimmunized mice did not increase around the OT II-T_H2 cell foci (see this article's Fig E3 in the Online Repository at www.jacionline.org). These results indicate that unprimed CD4 T cells do not accumulate in the foci formed by antigen-specific T_H2 cells. We have performed time course experiments. Most antigen-specific T_H2 cells accumulated in the lung within 24 hours after antigen inhalation. The accumulated cells that formed foci remained as long as 72 hours after antigen inhalation (data not shown).

Real-time cellular dynamics of T_H2-cell accumulation in the lung of living mice in a mouse model of asthma

Real-time cellular dynamics of antigen-specific T_H2-cell accumulation into the lung was then imaged in living mice at the cellular level by using scanning laser fluorescence microscopy. To image this process, antigen-specific T_H2 cells generated *in vitro* from naive CD4 T cells of GFP⁺ OT II Tg mice were transferred intravenously into normal C57BL/6 mice. The recipient mice were administered ovalbumin by inhalation. They were then

anesthetized and tracheostomized, and the lung was exposed microsurgically at various time points after ovalbumin inhalation. First, to assess any changes in blood flow rates in the clamped right lung and ventilated left lung, fluorescent microspheres were infused before and after clamping and ventilation. The lungs were then monitored by fluorescence microscopy (see this article's Fig E4 in the Online Repository at www.jacionline.org). Thirty minutes after clamping and ventilation, blood flow rates in both lungs were comparable. The relative blood flow was still kept at more than 80% of that before clamping and ventilation. Thus, the relative blood flow rates in the clamped lung appeared not to be changed in this experimental system.

The clamped right lungs were then imaged with the IV100 scanning laser microscope. Before ovalbumin inhalation, no foci were observed in the lung (Fig 2, B; Table I; see this article's Video E1 in the Online Repository at www.jacionline.org). The rate of rolling GFP⁺ T_H2 cells in the field was 14.7 ± 1.5 cells/mm²/30 min. Some of the rolling cells attached and accumulated in the field (7.0 ± 1.5 cells/mm²/30 min), and a similar amount of the T_H2 cells egressed from the field (7.0 ± 1.0 cells/mm²/30 min). These results suggest that the allergen-specific effector T cells were rolling in the lung vessels, and some were migrating into and accumulating in the lung, whereas some were migrating out of the lung. At time 0, the ratio of cells that accumulated and egressed from the field was equivalent, and the total cell number in the lung appeared to be maintained at a constant level. Only 10% of the T_H2 cells in the lung were crawling in this stable state (see this article's Video E2 in the Online Repository at www.jacionline.org). Six hours after ovalbumin inhalation, small foci were observed (Fig 2, B, red asterisks). By 6 hours, the number of rolling T_H2 cells greatly increased compared with the stable state (33.3 ± 3.1 vs 14.7 ± 1.5 cells/mm²/30 min; Table I). GFP⁺ T_H2-cell accumulation in the lung also increased (15.3 ± 1.5 cells/mm²/30 min; Table I; see this article's Video E3 in the Online Repository at www.jacionline.org). The degree of GFP⁺ T_H2-cell egress from the field was similar to that observed in the stable state. By 6 hours, the ratio of crawling cells was observed to increase (30.5% from 10.0%). These observations suggest that the allergen-induced migration and accumulation of T_H2 cells into the lung began to be upregulated by 6 hours after ovalbumin inhalation. The formation of foci was also observed approximately 6 hours after ovalbumin inhalation. Twelve hours after ovalbumin inhalation, the foci became larger, and the number of T_H2 cells rolling in the field further increased (44.7 ± 4.5 cells/mm²/30 min; Table I; see this article's Video E4 in the Online Repository at www.jacionline.org). T_H2-cell accumulation in the lung also further increased (24.3 ± 2.5 cells/mm²/30 min). However, the degree of T_H2-cell egress from the field did not obviously change compared with that observed in the stable state (8.3 ± 1.6 vs 7.0 ± 1.0). At 12 hours after ovalbumin inhalation, 90% of the T_H2 cells accumulating in the field were crawling (see this article's Video E5 in the Online Repository at www.jacionline.org). Thus, allergen-induced migration and accumulation of T_H2 cells into the lung appeared to be highly upregulated at approximately 12 hours after ovalbumin inhalation. At 21 hours after ovalbumin inhalation, nonfocal T_H2 cells greatly increased (868.7 ± 296.5 cells/mm²/30 min at 21 hours). More than 95% of the accumulating cells were crawling at this time point. However, at 21 hours, T_H2 cells rolling into the field were apparently reduced (2.7 ± 0.6

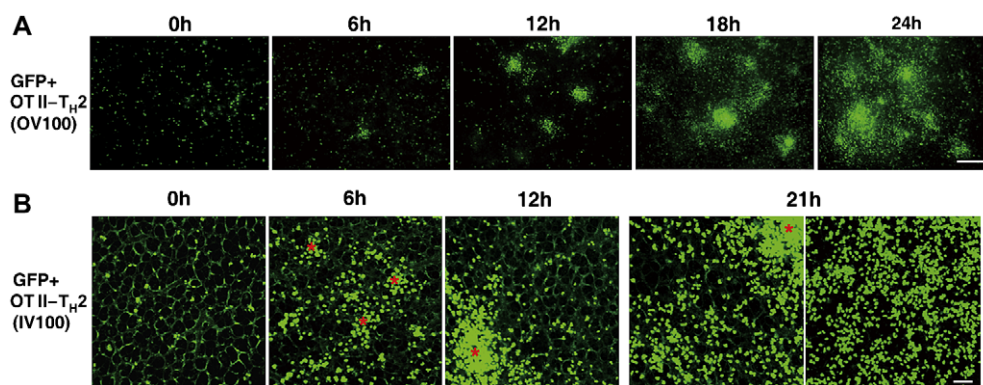


FIG 2. Imaging of ovalbumin-specific OT II- T_H2 -cell accumulation in the lung and the generation of GFP⁺ T_H2 -cell foci after ovalbumin inhalation. GFP⁺ OT II- T_H2 cells, differentiated *in vitro* from naive CD4 T cells from GFP⁺ OT II Tg mice, were intravenously transferred into C57BL/6 mice. Two days later, the recipient mice were administered ovalbumin by inhalation. The mice were subjected to imaging at indicated time points after ovalbumin inhalation. GFP⁺ T_H2 cells were imaged by using the OV100 Small Animal Imaging System (A) or the IV100 scanning laser imaging system (B). Red asterisks indicate the site of focus formation. Bar, 300 μ m (A) and 100 μ m (B). The results are representative of 5 (A) and 3 (B) experiments.

TABLE I. Summary of nonfocal OT II- T_H2 cell accumulation in the lung after ovalbumin inhalation

After ovalbumin inhalation	0 h	6 h	12 h	21 h
Cell number in nonfocal area (cells/mm ²)	57.7 $\pm$ 4.5	134.3 $\pm$ 16.9	226.0 $\pm$ 25.1	868.7 $\pm$ 296.5
Cells rolling in the blood vessel (cells/mm ² /30 min)	14.7 $\pm$ 1.5	33.3 $\pm$ 3.1	44.7 $\pm$ 4.5	2.7 $\pm$ 0.6
Cells accumulated in the field (cells/mm ² /30 min)	7.0 $\pm$ 1.5	15.3 $\pm$ 1.5	24.3 $\pm$ 2.5	1.3 $\pm$ 0.6
Cells egressed from the field (cells/mm ² /30 min)	7.0 $\pm$ 1.0	8.0 $\pm$ 1.5	8.3 $\pm$ 1.6	0.7 $\pm$ 0.6
Cells crawling in the field (%)	10.0	30.5	90.0	96.0

The data are presented as the mean cell number + SD from 3 independent experiments unless otherwise indicated.

cells/mm²/30 min; Table I; see this article's Video E6 in the Online Repository at www.jacionline.org). Therefore, the allergen-induced migration and accumulation of T_H2 cells into the lung reached maximum levels by 21 hours after ovalbumin inhalation. Between 6 and 12 hours after ovalbumin inhalation, the migrating T_H2 cells predominantly formed foci. Later, at 12 to 21 hours after ovalbumin inhalation, the T_H2 cells were observed to accumulate throughout the lung rather than form foci.

Effect of dexamethasone on the accumulation of ovalbumin-primed CD4 T cells in the lung in a mouse model of asthma

The color-coded imaging system was then used to determine the effect of dexamethasone, a potent drug that attenuates allergic reactions, on the accumulation of allergen-primed CD4 T cells in the lung. Dexamethasone (0.4, 1, or 4 mg/kg) was administered intraperitoneally 1 hour before ovalbumin inhalation. GFP⁺ CD4 T cells were monitored by fluorescence imaging 24 hours after ovalbumin inhalation. A marked dexamethasone dose-dependent decrease in the number of infiltrated CD4 T cells was observed in the excised lung (Fig 3, A and B). These results suggest that the efficacy of dexamethasone in inhibiting the development of ovalbumin-induced airway inflammation was, at least in part, a result of the inhibition of CD4 T-cell accumulation into the lung.

Dexamethasone was then administered intraperitoneally after ovalbumin inhalation (on day 16), and GFP⁺ CD4 T cells were monitored by fluorescence imaging 1 day later. The extent of infiltration of GFP⁺ CD4 T cells was again observed to decrease significantly (Fig 3, C and D). These results indicate that the infiltration of CD4 T cells was reduced if dexamethasone was administered even after the onset of airway inflammation.

Contribution of adhesion molecules to the generation of T_H2 -cell foci in the lung in a mouse model of asthma

We next assessed the contribution of adhesion molecules to the generation of T_H2 -cell foci in the lung in a mouse model of asthma. The blockage of ICAM-1 and VCAM-1 by antibodies to these molecules is known to inhibit airway inflammation.¹⁴ To test the role of ICAM-1 and VCAM-1 in our model, GFP⁺ OT II- T_H2 cells were transferred intravenously into normal C57BL/6 mice. One day before ovalbumin inhalation, mice were injected intraperitoneally with 200 μ g anti-ICAM-1 (YN1/1.7.4) or anti-VCAM-1 (429) mAbs. One day after ovalbumin inhalation, transferred ovalbumin-specific GFP⁺ T_H2 cells were monitored by using fluorescence imaging. The generation of GFP⁺ T_H2 -cell foci in the lung was substantially inhibited by treatment with anti-ICAM-1 and anti-VCAM-1 mAbs (Fig 3, E and F). Our model demonstrates that ICAM-1 and VCAM-1 play an essential role in focus formation by the control of

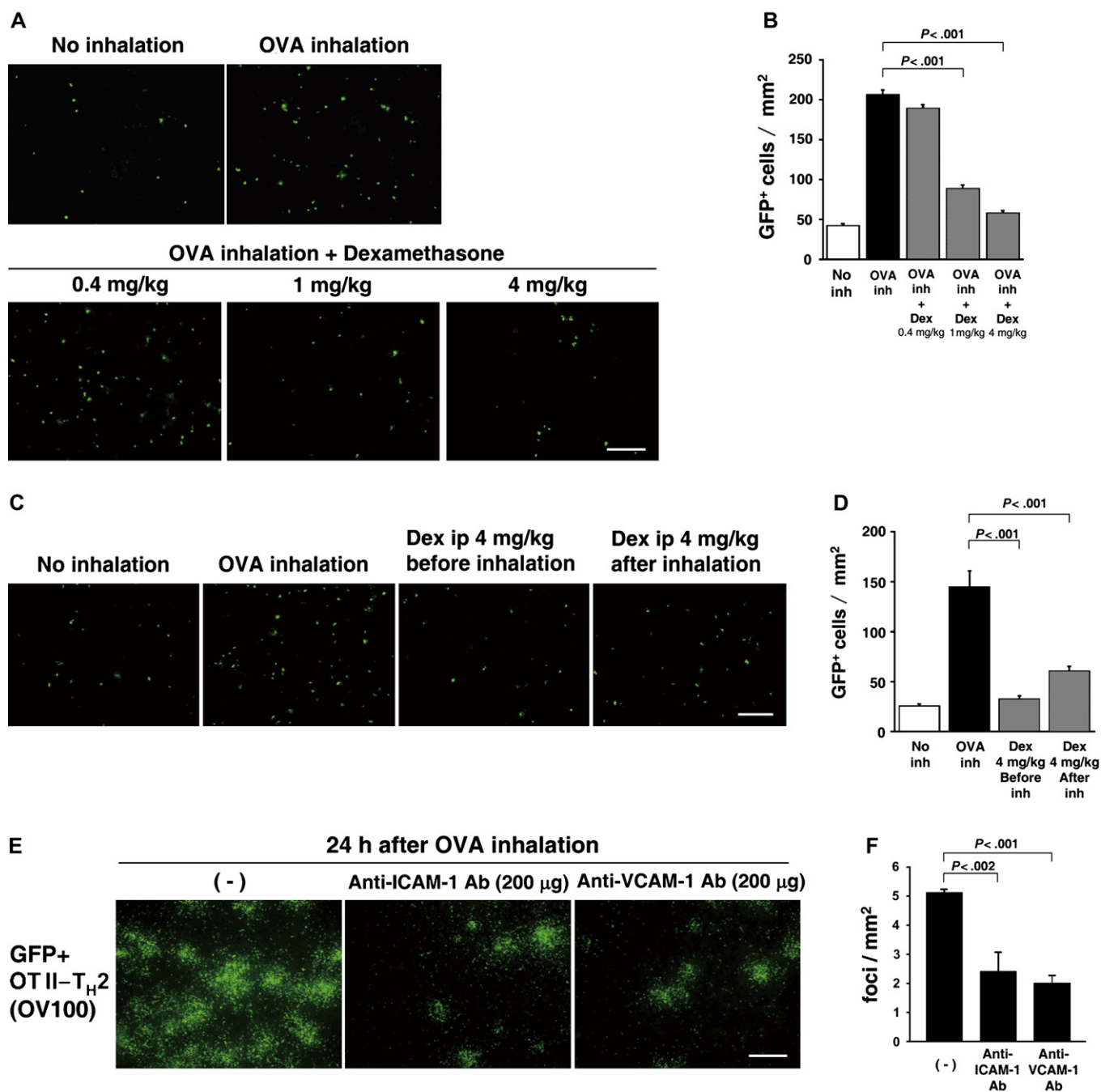


FIG 3. Effects of dexamethasone, anti-ICAM-1, and anti-VCAM-1 treatment on CD4 T-cell accumulation in the lung after ovalbumin (OVA) inhalation. **A**, Allergic airway inflammation was induced as in Fig 1, A. Three different doses of dexamethasone (DEX) were injected intraperitoneally 1 hour before OVA inhalation. Twenty-four hours after inhalation (day 16), GFP⁺ CD4 T cells were monitored by using the OV100 Small Animal Imaging System. Bar, 100 μm. **B**, Summary of the accumulation of fluorescent cells of A. Data are from 15 fields from 3 mice with SD. $P < .001$ by the Student's *t* test. **C**, Dexamethasone was injected intraperitoneally 1 hour before or 1 day after OVA inhalation. Two days after inhalation, GFP⁺ CD4 T cells were monitored in the excised lung by using the OV100 Small Animal Imaging System. Bar, 100 μm. **D**, Summary of the accumulation of fluorescent cells of C. The data are from 15 fields from 3 mice with SD. $P < .001$ by the Student's *t* test. **E**, Allergic airway inflammation was induced as in Fig 2, A. Anti-ICAM-1 or anti-VCAM-1 mAb was injected intraperitoneally 24 hours before OVA inhalation. Twenty-four hours after OVA inhalation, GFP⁺ OT II-T_H2 cells were monitored. Bar, 100 μm. **F**, Summary of the generation of T_H2-cell foci in E. Data are from 12 fields from 3 mice with SD. $P < .002$, $P < .001$ by the Student's *t* test.

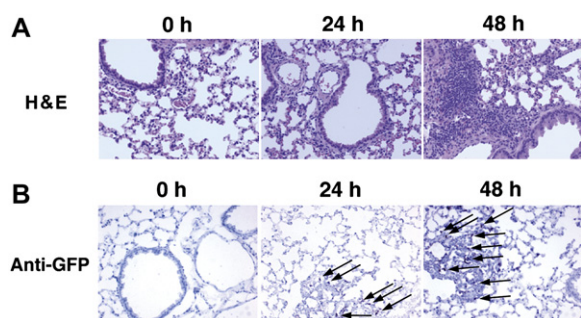


FIG 4. Eosinophilic infiltration and GFP⁺ TH₂-cell infiltration into the lung in a mouse model of asthma. **A**, The lung specimens were fixed at indicated time point after ovalbumin inhalation and stained with H&E. A representative H&E staining pattern in each group is shown. Magnification $\times 200$. **B**, Representative immunohistochemical staining for GFP is shown. Magnification $\times 200$. Arrows indicate some of the representative GFP-positive cells. The results are representative of 3 experiments.

TH₂-cell migration in the lung. Thus, TH₂ focus formation appears to be critical in the development of allergic airway inflammation in this animal model.

Eosinophilic infiltration and GFP⁺ TH₂-cell infiltration into the lung in a mouse model of asthma

Previous studies in animal models suggest a TH₂ paradigm for allergic diseases, with an increased activation of TH₂ cells that produce TH₂ cytokines, thereby resulting in the recruitment and activation of eosinophils. Marked eosinophilic infiltration is characteristic 2 or 3 days after allergen challenge in animal models of allergic airway inflammation.^{7,9,10} The foci formed by TH₂ cells observed in the current study and accumulation of eosinophils may be coincidental because our results showed that antigen-specific TH₂ cells accumulated and formed foci in the lung after allergen inhalation. GFP⁺ OT I-TH₂ cells were intravenously transferred into C57BL/6 mice, and 2 days later, the recipient mice were exposed to ovalbumin. Typical peribronchiolar and perivascular infiltration and focus formation by eosinophils were observed 48 hours after ovalbumin inhalation (Fig 4, A). The infiltrated OT II-TH₂ cells were monitored by immunohistochemistry analysis with an anti-GFP antibody. We observed GFP⁺ transferred TH₂ cells in the region of subsequent eosinophilic infiltration (Fig 4, B). These results, in conjunction with the results of our time course experiments (Fig 2), indicate that antigen-specific TH₂ cells accumulate and form foci in the lung before the marked infiltration of eosinophils and thus regulate the initiation of inflammatory processes in this animal model.

DISCUSSION

The behavior of T cells during airway inflammation in mouse models of asthma has been investigated by using flow cytometry and immunohistochemistry.²⁴⁻²⁸ These studies showed that T cells migrate into the lung after allergen challenge, but the studies did not address how and when they migrate into the lung or whether only antigen-specific effector T cells migrate into the lung. Several investigators performed lung imaging in a serial but static manner. Hutchison et al²⁹

have used serial tissue sectioning to describe the time course of proliferating CD4 T cells in the lung and its draining lymph nodes. Bhattacharya's group^{30,31,32} imaged whole excised lungs to study signaling by lung resident cells. Until now, the *in vivo* dynamics of cell invasion of the lung during inflammation in living animals has been poorly understood because it has been difficult to arrest motion in the lung as a result of the beating heart or movement during respiration. Our novel imaging model has overcome these problems and has demonstrated, for the first time, the dynamics of migration of allergen-specific TH₂ cells into the lung after allergen inhalation in living animals at the cellular level using the adoptive transfer of GFP⁺ T cells.

With this novel imaging model, several important findings are demonstrated. We have shown for the first time TH₂-cell focus formation in the lung, a cellular immunologic event occurring during the initiation of airway inflammation (Fig 2). In addition, we demonstrate that unprimed CD4 T cells and naive antigen-specific CD4 T cells did not accumulate around the foci (Fig 1; Figs E1-E3), indicating that the molecules specifically expressed on activated effector TH₂ cells play an important role in migration and focus formation. Moreover, TH₂-cell focus formation occurred before eosinophilic infiltration and thus may determine the eosinophilic inflammatory site (Figs 2 and 4). Focus formation was inhibited by the administration of anti-ICAM-1 and anti-VCAM-1 antibodies (Fig 3, E and F), both of which are able to block the induction of eosinophilic airway inflammation, indicating that focus formation is a critical process during the induction of the asthma phenotype.

Several groups have shown time-lapse microscopy of GFP-labeled and/or RFP-labeled cancer cells in live mice. Hoffman's group^{22,23} has demonstrated *in vivo* imaging of intracapillary and intralymphatic cancer cell trafficking behavior. Condeelis' group³³ has used *in vivo* imaging to determine molecular mechanisms of cancer metastasis.

Many groups have reported *in vivo* imaging of lymphoid tissues such as lymph nodes, spleen, and bone marrow to visualize antigen presentation and T-cell migration.³⁴⁻⁴¹ To visualize the cells *in vivo*, they must be labeled by appropriate dyes or express fluorescent proteins. In most of the reports of *in vivo* imaging of lymphoid tissues, T cells were labeled by using appropriate dyes and transferred into recipient mice, although a possible difference in the migration behavior of the cells that were labeled by dyes has been suggested.⁴² In our study, we prepared ovalbumin-specific TH₂ cells expressing GFP from GFP Tg mice¹⁶ and transferred the cells intravenously into normal recipient mice. Another technical issue could be the difference in the migration between antigen-specific cells that were injected into the mice before imaging and antigen-specific T cells that were resident in the imaged mice. In the near future, the behavior of antigen-specific TH₂ cells that were resident in the imaged mice will be investigated.

This model allows investigators to monitor the migration of inflammatory lymphocytes into the lung in a real-time manner in live animals and thus provides a new strategy to study the *in vivo* cell biology of inflammatory lung diseases such as asthma. This method can be also applied to various bacteria-induced and virus-induced inflammatory lung diseases, including tuberculosis and influenza virus-induced pneumonia, and to screen for more effective drugs for these respiratory diseases.

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Key messages

- We established a novel *in vivo* real-time color-coded cellular imaging model to visualize the dynamics of migration of T cells in the lung in a mouse model of asthma.
- Accumulating T_H2 cells formed foci in the lungs 6 to 20 hours after allergen inhalation.
- The focus formation was dependent on ICAM-1 and VCAM-1 and appeared to determine the site of eosinophilic infiltration.
- T_H2 -cell focus formation appears to be a critical process in the induction of allergic airway inflammation in this animal model.

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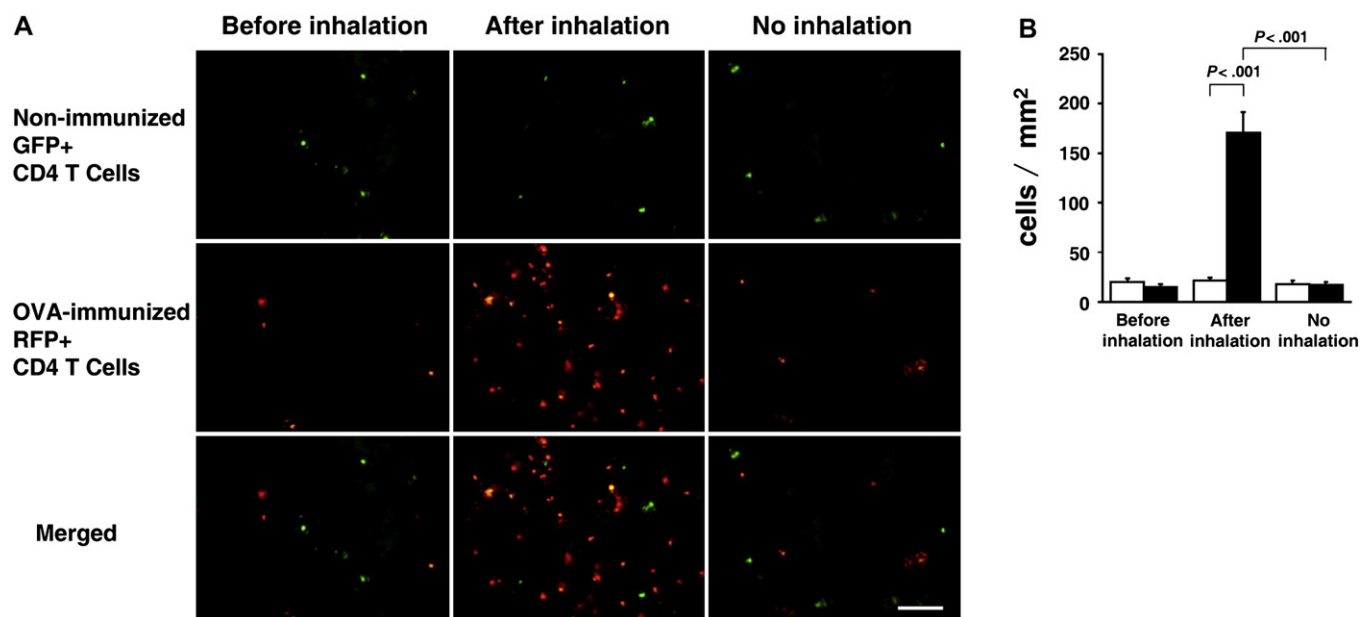


FIG E1. Color-coded fluorescence imaging of CD4 T-cell infiltration into the lung during ovalbumin (OVA)-induced allergic asthma. **A**, Color-coded images of CD4 T cells with the opposite color to the experiment shown in Fig 1, **B**. Splenic CD4 T cells from OVA-primed RFP Tg and nonprimed GFP Tg mice were injected into normal recipient mice. GFP⁺ and RFP⁺ CD4 T cells were monitored in the excised lung as in Fig 1, **B**. Bar, 100 μ m. **B**, Summary of the accumulation of fluorescent cells of **A**. Data are from 15 fields from 3 mice with SD. Open bar, GFP; closed bar, RFP. $P < .001$ by the Student's *t* test.

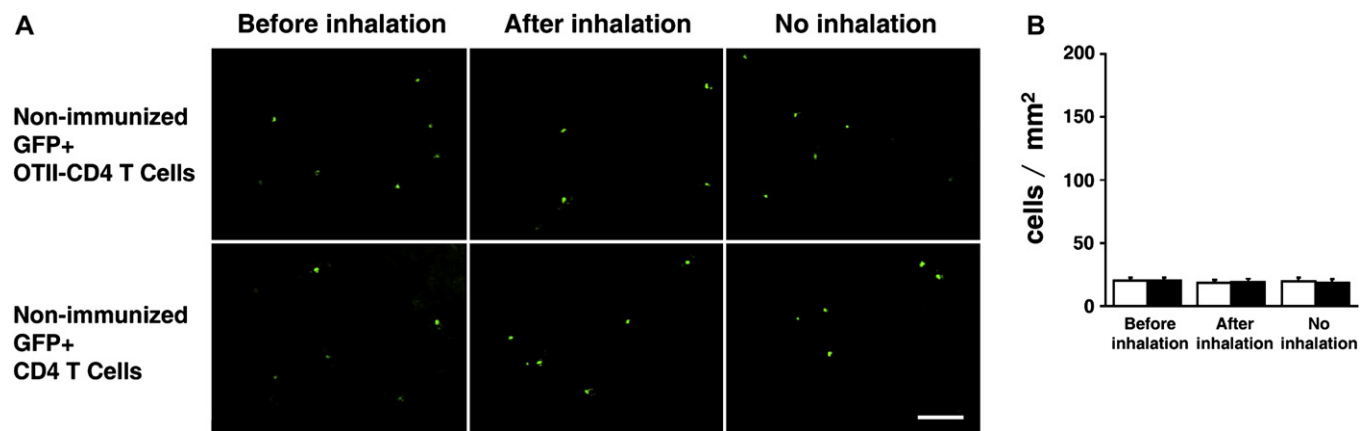


FIG E2. Fluorescence imaging of naive OT II-CD4 T and CD4 T cells in the lung. **A**, Images of naive OT II-CD4 T and CD4 T cells. Unprimed GFP⁺ OT II-CD4 T and CD4 T cells were monitored in the lung before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. Bar, 150 μ m. **B**, Summary of the accumulation of fluorescent cells of **A**. Data are from 15 fields from 3 mice with SD. *Open bar*, OT II-CD4 T cells; *closed bar*, CD4 T cells.

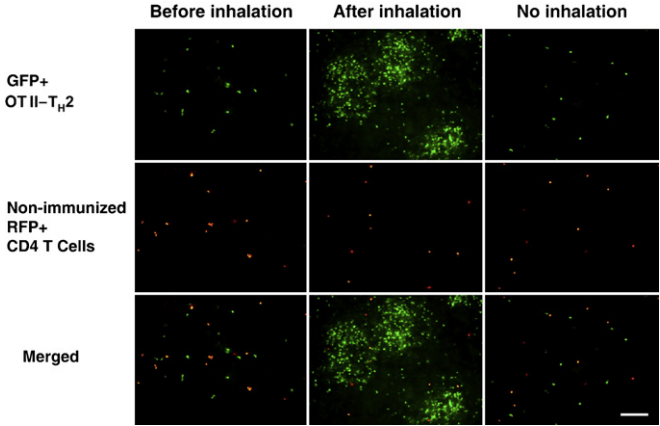


FIG E3. Color -coded fluorescence imaging of OT II-T_H2 cells and CD4 T cells in the lung. Primed GFP⁺ OT II-T_H2 cells and RFP⁺ CD4 T cells from nonprimed RFP Tg mice were monitored before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. Bar, 150 μm. The results are representative of 3 experiments.

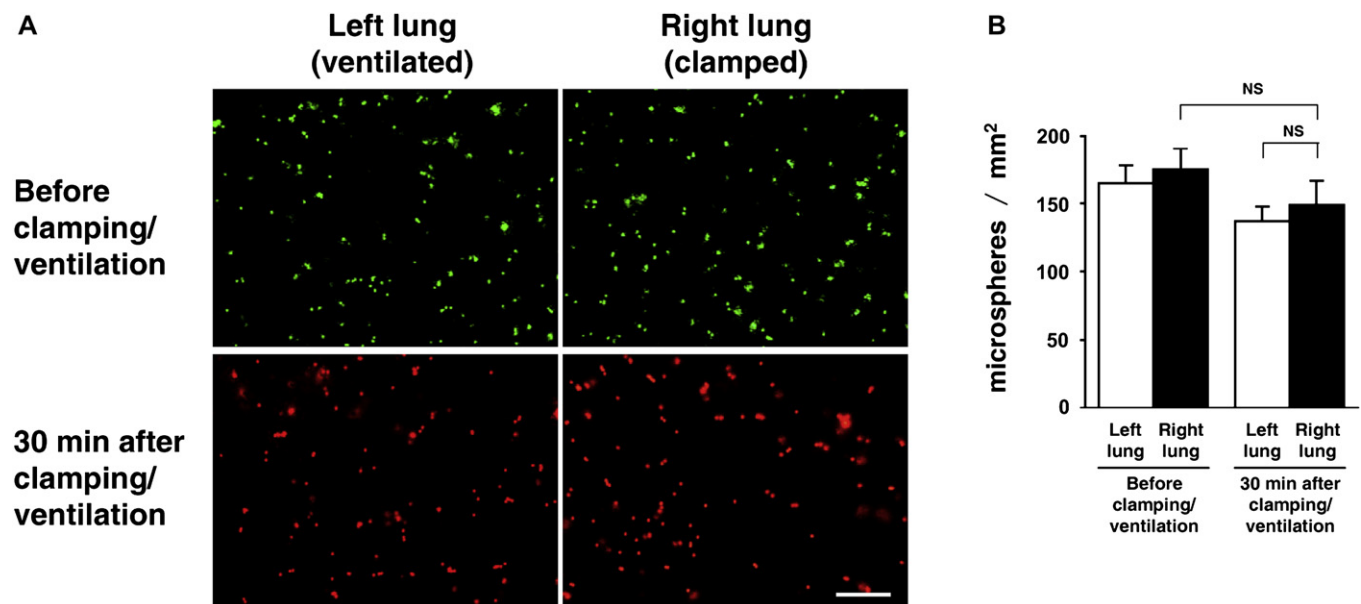


FIG E4. Measurement of blood flow in the clamped right lung and the ventilated left lung. **A**, Assessment of blood flow using fluorescent microspheres. Yellow-green fluorescent microspheres (10 μ m, Invitrogen) were infused before ventilation and red fluorescent microspheres (10 μ m, Invitrogen) infused 30 minutes after ventilation. The excised lungs were assessed by using the OV100 Small Animal Mouse Imaging System. Bar, 150 μ m. **B**, Summary of the number of fluorescent microspheres. Data are from 9 fields from 3 mice with SD. *Open bar*, left lung; *closed bar*, right lung *NS*, not significant.

VIDEO E1-E6. Dynamic real time cellular imaging of ovalbumin-specific GFP⁺ OT II-T_H2 cell migration into the lung after ovalbumin inhalation in living mice. Ovalbumin-specific GFP⁺ T_H2 cell migration and accumulation in the lung before ovalbumin inhalation (Videos E1 and E2 [high magnification]) and after ovalbumin inhalation: 6 hours (Video E3), 12 hours (Videos E4 and E5 [high magnification]), and 21 hours (Video E6). Individual cells were imaged every 5 seconds. Videos are at ×225 real-time.