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anti–Ser5-P RNAPII (Abcam), anti–Ser2-P RNAPII (Abcam), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), anti-Suz12 (Abcam), and anti-EZH2 (Diagenode). The antibodies used for EMSA were anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-CBP (Abcam), and anti-p300 (Millipore). The antibodies used for cytoplasmic staining were anti–IFN-γ–FITC, anti–IL-4–PE, and anti-GATA3–Alexa Fluor 647 (BD).

The generation of Th1 and Th2 cells. Th1/Th2 cells were generated as previously described (Yamashita et al., 2006). In brief, splenic CD4 T cells were stimulated with 3 μg/ml of immobilized anti–TCR-β mAb plus 1 μg/ml anti-CD28 mAb under the Th1 or Th2 culture conditions for 5 d in vitro. Th1 conditions were as follows: 25 U/ml IL-2, 10 U/ml IL-12, and anti–IL-4 mAb. Th2 conditions were as follows: 25 U/ml IL-2 and 100 U/ml IL-4. These cells were used as either Th1 or Th2 cells.

Establishment of fully developed Th2 cells. Splenic CD4 T cells from DO11.10 OVA-specific TCR transgenic mice were stimulated with an OVA peptide (1 μM Loh15) plus APC under Th2 culture conditions for 5 d in vitro. The Th2 cells were further cultured in vitro for 2 d in the absence of any exogenous cytokines. The cultured CD4 T cells were then restimulated with OVA peptide (1 μM Loh15) plus APC with IL-2 and anti–IL-4 mAb for 5 d. This cycle was then repeated more than three times.

Quantitative RT-PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen). cDNA was synthesized using oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative RT-PCR was performed as described previously using a sequence detection system (ABI Prism 7500; Applied Biosystems; Yamashita et al., 2006). The primers and TaqMan probes for the detection of *GATA3*, Bmi1, EZH2, Menin, MLL, and HPRT were purchased from Applied Biosystems and Roche, respectively. The specific primers and Roche Universal probes used are described in Table S1. The expression was normalized by the HPRT signal.

ChIP assay. ChIP was performed using ChIP assay kits (Millipore) as previously described (Yamashita et al., 2006). Quantitative representations of the results are shown as relative band intensities measured by a densitometer (AE6905H [ATTO] and CS Analyzer version 2.08b). The specific primers used are described in Table S1. Real-time quantitative PCR analysis was performed on an ABI Prism 7500 real time PCR machine with TaqMan probes and primers (sequences available in Table S1). To calculate the enrichment of each protein to a particular target DNA, values obtained (via the standard curve method) for each target were divided by the amount of the corresponding target in the input fraction. Enrichments obtained from mock immunoprecipitations performed in parallel with normal IgG were then subtracted from the enrichment values obtained with specific antibodies ([specific antibody ChIP − control Ig ChIP]/input DNA). All the enrichments are expressed as a function of the highest enrichment obtained on the locus (set to 10; Demers et al., 2007).

EMSA. EMSAs were performed using a gel shift assay system (Promega) as described previously (Kimura et al., 2005). In brief, the nuclear extracts were incubated at room temperature with a ^{32}P-labeled, double-stranded oligonucleotide in DNA-binding buffer. In some experiments, the nuclear extracts were preincubated at 4°C with specific antibodies. Electrophoresis was conducted on 4% native polyacrylamide gel (acrylamide/bisacrylamide ratio 29:0.8 in 0.5× Tris-borate-EDTA), and the radioactivity was visualized by autoradiography. The oligonucleotides used in this experiment were normal probe S4, 5′-CTTGGCGTTCAAGAGAATTCTCAA-3′; mutant probe S4, 5′-CTTGGCGTTC***GGTTT***AATTCTCAA-3′; normal probe S7, 5′-AGCCAACTTCCTAGGAAAAAGCTG-3′; and mutant probe S7, 5′-AGCCAACTTC***AGTTT***AAAAAGCTG-3′. The STAT6 consensus motif is underlined, and the mutated nucleic acids are shown in italicized and bold characters.

TSA treatment. STAT6-deficient splenic CD4 cells were cultured under Th2 conditions, and 10 nM TSA (Sigma-Aldrich) was added in the culture on day 2. After another 3-d culture, CD4 T cells were collected for the ChIP assay and RT-PCR.

Retroviral vectors and infection. The pMX-IRES-hNGFR plasmid was generated as previously described (Shinnakasu et al., 2006). Retrovirus vectors containing STAT6VT cDNA (pMXs-STAT6VT-IRES-hNGFR; Daniel et al., 2000) or STAT6VT dDBD cDNA (pMXs-STAT6VT-dDBD-IRES-hNGFR) were used. The infected cells were enriched by magnetic cell sorting with anti-hNGFR (clone C40-1457; BD) and were subjected to a ChIP assay.

A resting and restimulation culture system. Naive CD4 T cells from Menin-deficient mice were cultured under Th2 conditions for 5 d. The Th2 cells were further cultured for 2 d in the absence of cytokines added and then restimulated with anti-TCR mAb in the presence of IL-2 and anti–IL-4 mAb for an additional 5 d. This cycle was then repeated. PcG/TrxG binding and histone modifications were detected by ChIP assays, and the *GATA3* expression was determined using quantitative RT-PCR at the end of the each cycle.

Immunoblot analysis. Cytoplasmic extracts and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The antibodies used for the immunoblot analysis were anti–histone H3 (Abcam), anti-Erk1 (Santa Cruz Biotechnology, Inc.), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), and anti-EZH2 (Diagenode).

ChIP-seq and Illumina sequencing. For ChIP-seq analysis, immunoprecipitate and input samples were prepared using ChIP-Seq Sample Prep kit (Illumina, Inc.). Adaptor-ligated DNA fragments were size-fractionated by 12% acrylamide gel, and the 150–250-bp fraction was recovered. DNA thus obtained was amplified by 18 cycles of PCR. 1 ng of the DNA was used for the sequencing reaction of the GAIIx (Illumina, Inc.) according to the manufacturer's instructions. 150,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences (mm9 as from the University of California, Santa Cruz Genome Browser) using the Eland program. Sequences allowing no more than two mismatches per sequence were used for the analysis.

Online supplemental material. Fig. S1 shows the real PCR product bands of the results shown in Fig. 1 C. Fig. S2 shows expression of Bmi1, EZH2, Menin, and MLL in naive CD4 T cells, Th2 cells, and fully developed Th2 cells. Fig. S3 shows the binding pattern of phosphorylated RNAPII at the *GATA3* gene locus. Fig. S4 shows that expression of Bmi1, EZH2, Menin, and MLL was not affected by TSA treatment in the STAT6-deficient Th2 cells. Fig. S5 shows phenotypic characterization of peripheral CD4 T cells in Menin-deficient mice. Fig. S6 shows the levels of total H3 at the *GATA3* gene locus in Menin-deficient Th2 cells. Fig. S7 shows IL-4/STAT6-independent maintenance of *GATA3* expression in developed Th2 cells. Fig. S8 shows binding of Menin protein to specific regions around the *GATA3* gene locus. Table S1 shows primers and probes used for ChIP and RT-PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100760/DC1.

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Current Opinion in Immunology

Role of NKT cells in allergic asthma

Chiaki Iwamura and Toshinori Nakayama

T helper 2 (Th2) cells play crucial roles in the development of allergic asthma, while various distinct cell populations also contribute to the pathogenesis of the disease. Invariant natural killer T (iNKT) cells produce large amounts of cytokines such as IL-4 and IFNγ upon stimulation with a ligand, α-galactosylceramide, and regulate various immune responses. Recently, a critical role of iNKT cells in the mouse model of asthma and also in asthma patients has been reported, while some contradictory results have also been described. Here, we summarize the experimental results in mouse and human systems, and discuss the current understanding of the role of NKT cells in the pathogenesis of asthma, including a possible mechanism by which iNKT cells are activated in asthma patients.

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Introduction

It is well established that $CD4^+$ T helper 2 (Th2) cells play an important role in allergic disorders by producing Th2 cytokines [1]. IL-4 induces antigen-specific IgE production from B cells, and IgE and antigen activate mast cells to release various chemical mediators. IL-5 induces the development, activation and survival of eosinophils, while IL-13 induces airway hyperreactivity (AHR) and mucus hyper production [2]. Animal studies investigating these processes are very helpful to explain the mechanisms underlying the development of allergic asthma. However, in asthma patients, several types of therapies targeted to these Th2 cytokines were not as effective as many investigators expected [3]. Indeed many patients show a non-allergic form of asthma in which no allergen-specific IgE are detected. Furthermore, it is well known that Th2 cell-independent factors such as viruses, air pollution, and exercise induce and/or exacerbate asthma symptoms. In addition, it has been reported that non-Th2 factors such as IFNγ, IL-17, and neutrophils contribute to severe asthma and steroid-resistant asthma [4,5]. These results indicate that Th2 responses are not obligatory in the development of asthma.

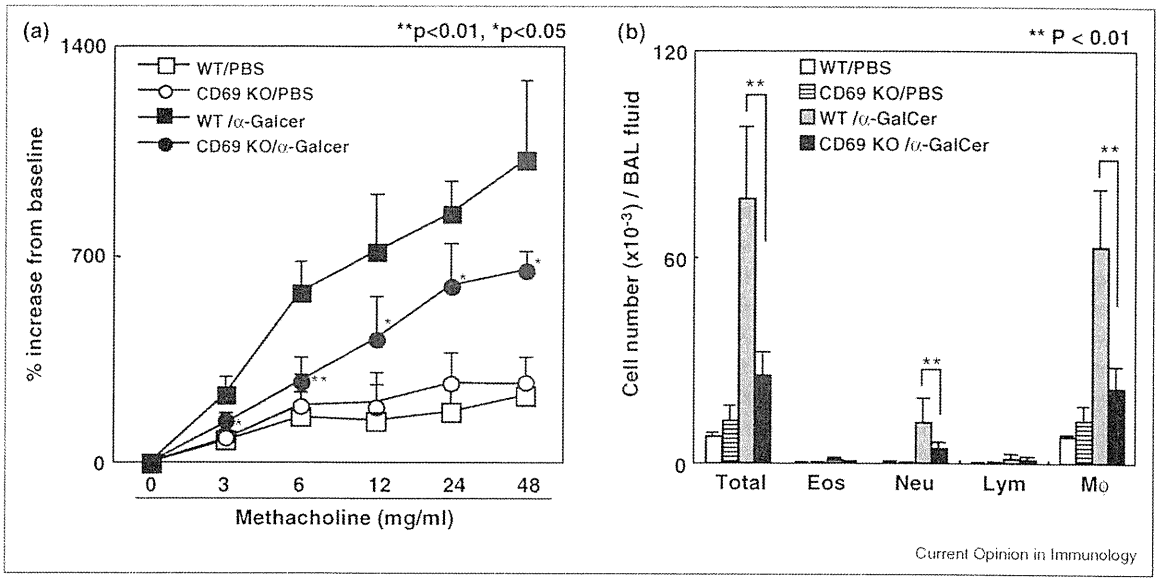
Natural killer T (NKT) cells belong to a novel lymphoid lineage distinct from T cells, B cells or NK cells. NKT cells are characterized by the expression of a restricted repertoire of T cell receptors (TCRs) consisting of Vα14 and Jα18 (in mice) or Vα24 and Jα18 (in humans) [6]. These receptors are associated with a highly skewed set of Vβs, mainly Vβ8.2 (in mice) and Vβ11 (in humans). Since these TCRs are highly restricted, NKT cells having these receptors are called invariant NKT cells (iNKT cells). The most potent and well-analyzed ligand for the iNKT antigen receptor is a glycolipid, α-galactosylceramide (α-GalCer), which is exclusively presented by CD1d, a monomorphic class Ib molecule. Activated iNKT cells play critical roles in the regulation of various immune responses, such as allergic inflammation, anti-tumor immunity and autoimmune responses.

Upon activation, iNKT cells produce a large amount of both type 1 and type 2 cytokines, that is, IFNγ and IL-4/IL-5/IL-13. Therefore, iNKT cells may inhibit or exacerbate allergic responses. The activation of iNKT cells with α-GalCer at the sensitization phase attenuated allergic airway inflammation and Th2 responses via production of IFNγ [7,8,9•]. In these reports, iNKT cells showed a suppressive function rather than promoting disease. On the other hand, many investigators have examined whether experimental allergic asthma is induced in iNKT cell-deficient mice or whether the activation of iNKT cells in the lung results in the development of asthma symptoms in mice. iNKT cells from patients with asthma were also assessed to reinforce the relationship between iNKT cells and allergic disease. In human studies, both positive and negative results regarding the contribution of iNKT cells in asthma were reported [10••,11••]. We herein focus on the role of iNKT cells in the pathogenesis of asthma in animal models and discuss the contribution of iNKT cells to human asthma.

Do NKT cells induce experimental allergic asthma?

Activated iNKT cells may induce allergic asthma symptoms independently from Th2 cells. To address this question, Akbari *et al.* assessed OVA-induced airway heperreactivity (AHR) and airway inflammation in iNKT cell deficient Jα281 KO and CD1d KO mice. This study

Figure 1



iNKT cell-dependent AHR and airway inflammation were attenuated in CD69-deficient mice. To examine the role of $CD69^+$ iNKT cells in NKT cell-dependent asthma, 1.5 μg of α-GalCer was administrated intranasally into wild type mice (WT) and CD69-deficient (KO) mice. **(a)** AHR in WT and CD69 KO mice 24 hours after α-GalCer treatment. Airway resistances were assessed in an invasive system as described previously [46]. **(b)** Decreased infiltrated leukocytes in BALF of CD69 KO mice after intranasal administration of α-GalCer. 24 hours after α-GalCer treatment, BALF of WT and CD69 KO mice were collected. Mean values (5 mice per group) are shown with SDs. $^*P < 0.05$ and $^{**}P < 0.01$, student *t* test.

showed a significant defect in the development of AHR and inflammation in the absence of iNKT cells [12]. The authors concluded that iNKT cells are required for the development of AHR and airway inflammation. The administration of anti-CD1d antibodies or a CD1d-dependent antagonist can also suppress OVA-induced AHR and airway inflammation [13,14•]. The respiratory administration of α-GalCer rapidly induced AHR and inflammation in naïve mice and this effect was seen even in naïve MHC class II-deficient mice, which lack conventional CD4 T cells [15]. In addition, a particular subset of iNKT cells that lack the NK1.1 marker produced IL-17 and induced neutrophilic infiltration following the intranasal administration of α-GalCer [16]. We recently reported that $CD69^+$ Th2 cells play a crucial role in OVA-induced airway inflammation and AHR [17]. As shown in Figure 1, α-GalCer-induced AHR and leukocyte infiltration, including neutrophilic infiltration was attenuated significantly in CD69-deficient mice, indicating that activated iNKT cell-mediated asthmatic responses were dependent on CD69. Thus, $CD69^+$ iNKT cells may play a critical role in the development of AHR and airway inflammation. Matangkasombut *et al.* demonstrated that the direct activation of pulmonary iNKT cells with α-GalCer in non-human primates resulted in the development of AHR, indicating that pulmonary iNKT cells are critical effector cells in this model [18].

However, there are several reports indicating that iNKT cells are dispensable for allergic airway inflammation. Allergic inflammation occurred normally under certain conditions in CD1d-deficient mice and β2-microgloblin (β2m) KO mice that lack iNKT cells [19]. More recently, however, Koh *et al.* found that AHR can develop in β2m KO mice [20]. In this report, they concluded that non-classical NKT cells, which are restricted to a β2m-independent form of CD1d contribute to the development of AHR. Although the reason is not clear, these discrepancies in the results obtained from β2m KO mice may be due to the difference in the experimental systems used.

iNKT cells in asthma patients

In order to identify the possible role of iNKT cells in human asthma, several investigators assessed the number of iNKT cells in asthma patients (Table 1). An initial report was published in 2006 by Akbari *et al.* reporting that more than 60% of CD4 T cells in the bronchoalveolar lavage fluid (BALF) from asthmatic patients were iNKT cells, while NKT cells were not observed in patients with sarcoidosis or in healthy controls [21]. Two other groups published supportive reports in the same year [22–24].

However, four other groups performed similar studies and concluded that iNKT cells did not increase in the patients with asthma [25–29]. Vijayanand *et al.* reported that iNKT cells were found in low numbers in the airways of patients with asthma, COPD or healthy controls with no significant differences between the three groups [26]. Mutalithas *et al.* also reported similar results in the BALF [27]. Moreover, the influx of iNKT cells into the airways

Table 1

iNKT cells in patients with asthma

Year	iNKT cells are			
	pathogenic		not pathogenic	
2006	Akbari	About 60% of $CD4^+CD3^+$ cells in BALF from patients with severe asthma were CD1d-restricted NKT cells [21]	Thomas	Only 0.4–2.1% of lymphocytes in BALF of asthmatic patients were 6B11+ NKT cells [25]
	Pham-ti	The frequency of CD1d-restricted NKT cells in BALF from severe asthmatic children was higher than in BALF from controls [22,23]		
	Hamzaoui	The number of $CD3^+CD56^+$NKT cells in the sputum of severe asthmatic patients is increased as compared to that of controls [24]		
2007			Vijayanand	Fewer than 2% of CD4T cells were CD1d-restricted, $6B11^+$ or $V\alpha24^+$ NKT cells in airway biopsy, BALF, and sputum of mild or moderately severe asthmatic patients and controls [26]
			Mutalithas	The presence of $6B11^+$ NKT cells in BALF of mild asthmatic patients was not significantly higher than that of controls [27]
			Thomas	Approximately 1% of lymphocytes were $6B11^+V\alpha24^+$NKT cells in BALF and this did not increase with an antigen challenge [28]
			Bratke	Less than 1% of T cells were $6B11^+$NKT cells in BALF of mild asthma patients [29]
2009	Matangkas ombut	CD1d-restricted NKT cells in BALF of severe or mild asthmatic patients increased (readdressed) [30••]		
	Reynold	CD1d-restricted NKT cells increased in lung specimens from mild asthmatic patients as compared to those from controls [31]		
2010	Koh	$V\alpha24^+$ or $6B11^+$ NKT cells were significantly increased in sputum from patients with asthma as compared to those from controls [32]		

BALF, bronchoalveolar lavage fluid.

was not observed after segmental allergen challenge [28,29].

By contrast, all studies we have identified from 2008 emphasized the existence of, and an increase in iNKT cells in patients with asthma [30••,31,32]. Matangkasombut *et al.* readdressed the issue regarding the number of iNKT cells in BALF from patients with severe asthma [30••]. They confirmed that patients with severe asthma had a significant increase in the number of iNKT cells as compared to healthy controls. In this report, however, the numbers of iNKT cells were 2–7% of total $CD3^+$ cells in BALF of asthmatic patients. Only one patient with severe asthma had 64.5% NKT cells. Reynolds *et al.* supported this idea using lung biopsies with allergen challenge [31]. At present, it is unclear why studies on iNKT cells in asthma patients have provided divergent results. However, as the filed matures it is becoming more evident that iNKT cells likely do play a role in the development and possibly exacerbation of allergic asthma. In addition, the studies of iNKT cells in other asthma etiologies, such as chronic, occupational, steroid-resistant, exercise-induced, and aspirin-induced asthma, where Th2 cells may not play a major role, could provide new insights into these types of diseases.

What activates iNKT cells *in vivo*?

Even if activated iNKT cells can cause and exacerbate allergic asthma, α-GalCer is a component of a marine sponge and it is very rare to be exposed to α-GalCer during daily life. Respiratory organs are continually exposed to environmental stimuli including allergens, pathogens, and air pollution. Several studies indicate that substances naturally occurring in our environment may activate iNKT cells and develop or exacerbate allergic airway inflammation.

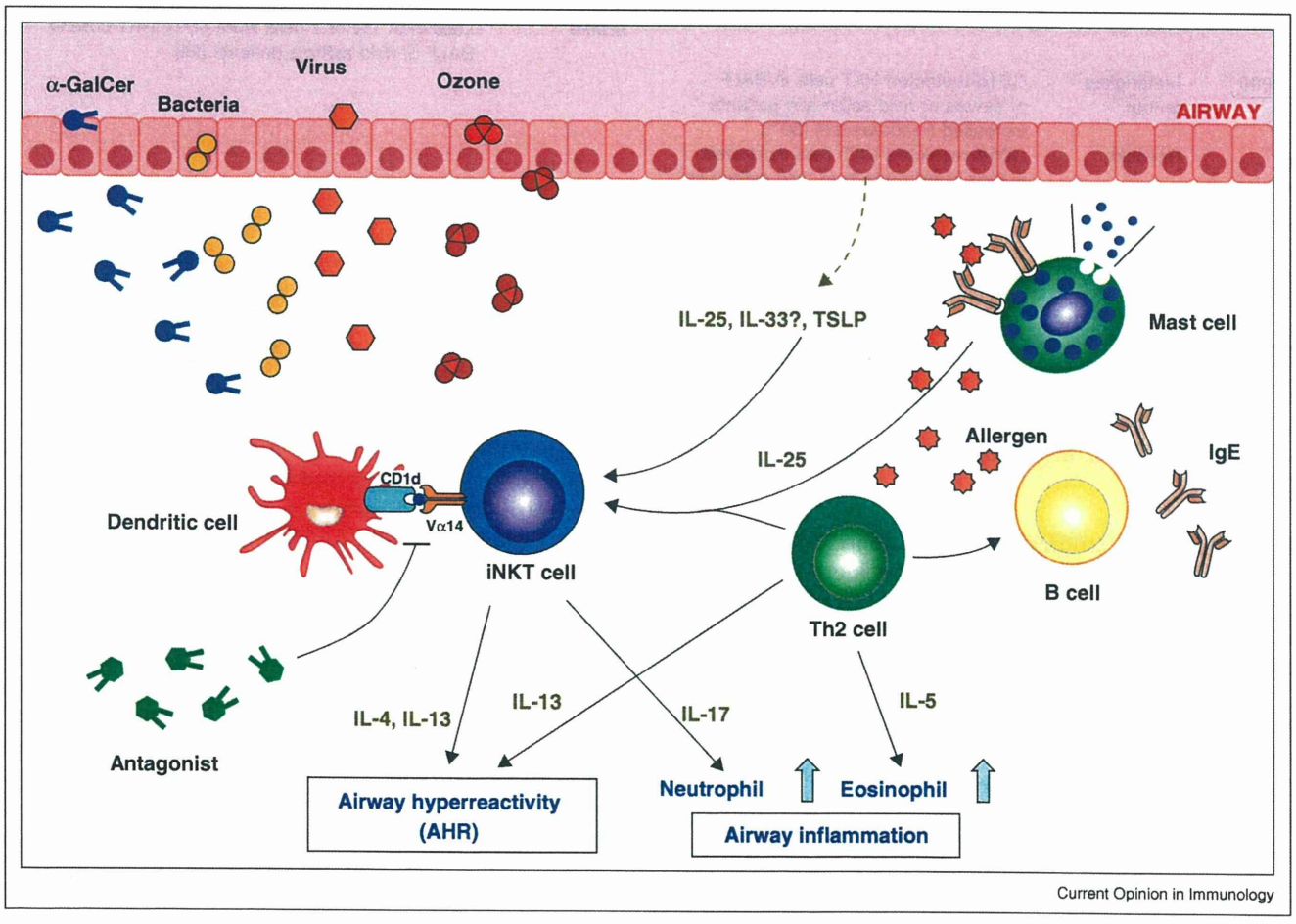
The invariant TCR of iNKT cells recognizes glycolipids from bacteria such as *Sphingomonas*, *Borrelia*, and *Leishmania* species [33]. Glycolipids purified from *Sphingoma-*

nas cell wall induced rapid AHR after respiratory administration in WT mice but not iNKT-deficient mice [15]. Once iNKT cells are activated by virus antigen, they stimulated macrophages to produce IL-13, resulting in the development of AHR and mucus production independently from the adaptive immune response [34]. It is well known that respiratory infection exacerbates the symptoms of allergic diseases under certain conditions. Therefore, iNKT cells activated by glycolipid from pathogens may contribute to the development and exacerbation of asthma symptoms in humans.

Ozone, as an air pollutant, has been reported to be associated with asthma [35,36]. Even in healthy individuals, exposure to ozone resulted in the development of AHR, associated with airway epithelial cell damage and increased numbers of neutrophils. Asthmatic patients appeared to be more susceptible to the adverse effects of this pollutant. Pichavant *et al.* showed that iNKT cells were required for ozone-induced asthma through production of IL-17 in the mouse lung [37]. Although it is not clear how ozone activates iNKT cells, NKT cells activated by ozone can induce a form of asthma that is characterized by neutrophilic infiltration and AHR.

Recently, several cytokines involved in the initiation and amplification of Th2 responses were reported [38]. IL-25 also known as IL-17E, a member of the structurally related IL-17 cytokine family, is produced by activated Th2 cells, epithelial cells, basophils, and mast cells and is capable of enhancing AHR. Administration of recombinant IL-25 induced Th2 type responses, including increased serum IgE levels, eosinophilia, pathological changes in the lung, and AHR. These symptoms induced by IL-25 were not observed in iNKT cell deficient mice [39•,40]. Transfer experiments of iNKT cells showed that iNKT cells expressing IL-17 receptor B (IL-17RB) are essential for IL-25-induced AHR. Thymic

Figure 2



Activated iNKT cells and Th2 cells in the development of AHR and airway inflammation. Th2 cytokines produced by antigen-specific Th2 cells can cause allergic asthma phenotypes through IgE induction, mast cell activation, eosinophilic infiltration into the lung, and AHR. Lung iNKT cells are activated by environmental substances or pro-Th2 cytokines. The activated iNKT cells induce AHR and infiltration of neutrophils predominantly in the airway by producing IL-13 and IL-17, respectively.

stromal lymphoprotein (TSLP) is produced by epithelial cells, mast cells, and basophils, and is considered to play an important role in experimental models of asthma. The targets of TSLP are T cells, mast cells, basophils and dendritic cells (DCs). In addition, Nagata *et al.* showed that TSLP also acts on iNKT cells to enhance AHR by up-regulating their production of IL-13 [41]. IL-33 is an IL-1 family member whose expression is also increased in epithelial cells in asthmatic patients. IL-33 enhanced the production of Th1 and Th2 cytokines in activated iNKT cells [42,43]. Based on these results, natural ligands in the environment may activate iNKT cells to induce allergic asthma, and pro-Th2 cytokines such as IL-25, TSLP and IL-33 may exacerbate the allergic symptoms.

How do iNKT cells control Th2 immune responses?

One enigma is the dynamics of iNKT cell accumulation in the lung during airway inflammation. Since the number of iNKT cells in the lung, lymphoid organs and PBMCs of humans and mice is very low (<0.1%) as compared to that of T cells or NK cells [44], it is curious how iNKT cells contribute to asthmatic symptoms. It is not known whether iNKT cells are activated and proliferated in the asthmatic lung or if activated iNKT cells migrate from lymphoid organs to the lung. We recently visualized the migration of antigen-specific Th2 cells into the lung in living mice after antigen exposure in the airway [45]. Using this *in vivo* live imaging system, visualization of the migration and localization of iNKT cells in the lung may provide important insights into these questions. In addition, possible cell–cell interactions between iNKT cells and various types of APCs, epithelial cells or Th2 cells in the asthmatic lung would be another interesting issue that may be clarified using *in vivo* imaging systems.

Conclusion

Figure 2 illustrates the role of activated iNKT cells and Th2 cells in AHR and airway inflammation. Both activated iNKT cells and Th2 cells produce various cytokines in the lung and induce AHR and airway inflammation. In the murine experimental model of asthma, activated iNKT cells appear to contribute as effector cells and also as an amplifier of allergen-specific Th2 cell responses. Although these mechanisms clarified in mouse models have not been directly investigated in asthmatic patients, NKT cells may yet prove to be a new therapeutic target for allergic diseases.

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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$_H$2 cells in the pathogenesis of acute asthma has been demonstrated in the studies of human asthma as well as of animal models of asthma. T$_H$2-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$_H$2 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$_H$2 cells formed foci in the lungs from 6 to 20 hours after antigen inhalation. This process was also inhibited by the administration of anti–intercellular adhesion molecule 1 or anti–vascular cell adhesion molecule 1 mAbs. Two days after inhalation of antigen, GFP$^+$ T$_H$2 cells were detected in the area of eosinophil infiltration.

Conclusion: Focus formation generated by accumulating antigen-specific T$_H$2 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 2010;125:461-8.)

Key words: *Real-time in vivo cellular imaging, cellular dynamics, T$_H$2 cells, mouse model of asthma, focus formation, airway inflammation, GFP, ICAM-1, VCAM-1*

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

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$_H$2 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$_H$2 cells formed foci in the lungs 6 to 20 hours after allergen inhalation. Focus formation was dependent on ICAM-1 and VCAM-1 and appeared to determine the site of eosinophilic infiltration, indicating that T$_H$2-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-FM131Osb (GFP Tg, C57BL/6 background) mice expressed an enhanced GFP in all tissue under the

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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 αβ transgenic (OT II Tg) mice[19] 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 μmol/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 μg 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 vain 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 CO_2 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 μg 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 O_2 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 μm 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-dimentional 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 CO_2 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 ×50 or ×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-μm-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 μg/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 (DAKOCytomation). 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 nonspecifically 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 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

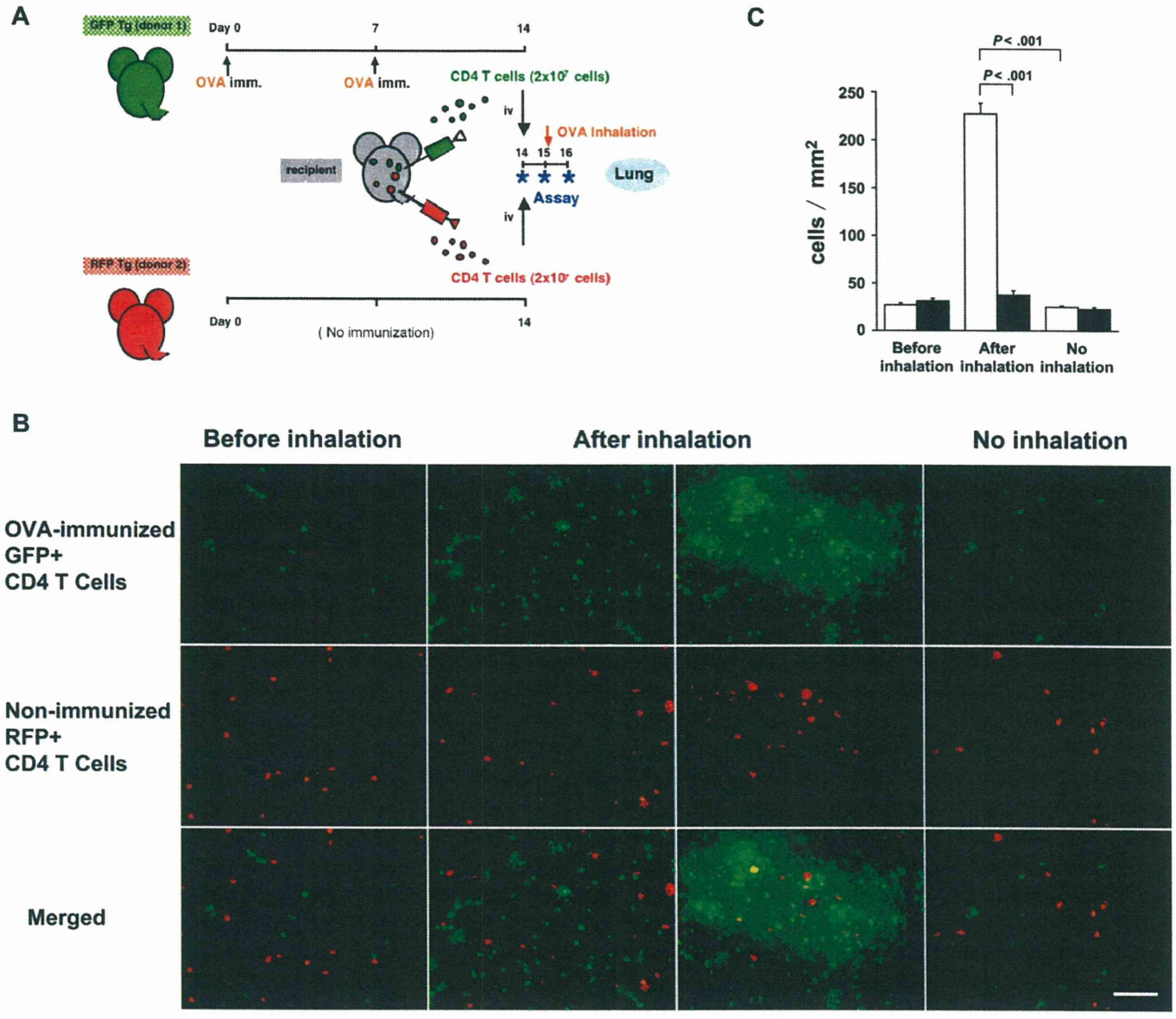

FIG 1. Color-coded fluorescence imaging of ovalbumin *(OVA)*-primed CD4 T-cell accumulation in the lung in the OVA-induced acute asthma model. **A,** A schematic overview of the study protocol for the induction of asthma. GFP Tg mice were sensitized with OVA on days 0 and 7. Splenic CD4 T cells from 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 μm. **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.

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 αβ 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/mm^2/30 min. Some of the rolling cells attached and accumulated in the field (7.0 + 1.5 cells/mm^2/30 min), and a similar amount of the T_H2 cells egressed from the field (7.0 + 1.0 cells/mm^2/30 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/mm^2/30 min; Table I). GFP^+ T_H2-cell accumulation in the lung also increased (15.3 + 1.5 cells/mm^2/30 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/mm^2/30 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/mm^2/30 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/mm^2/30 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 cells/mm^2/30 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

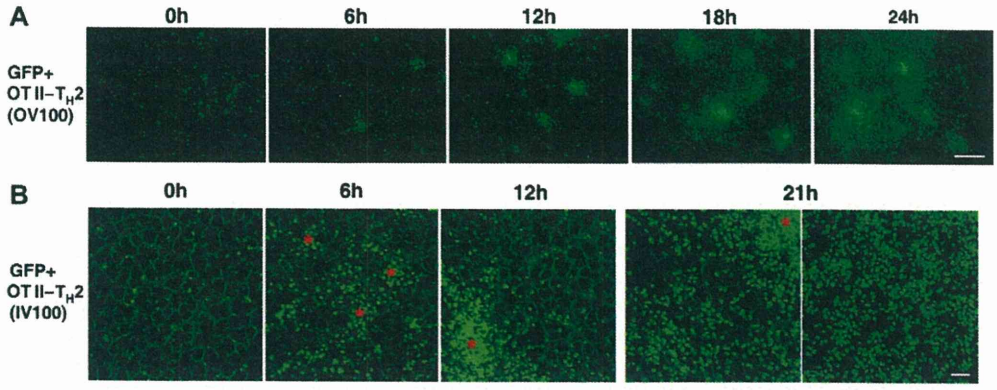

FIG 2. Imaging of ovalbumin-specific OT II–T_H2-cell accumulation in the lung and the generation of GFP^+ T_H2-cell foci after ovalbumin inhalation. GFP^+ OT II–T_H2 cells, differentiated *in vitro* from naive CD4 T cells from GFP^+ OT II Tg mice, were intravenously transferred into C57BL/6 mice. Two 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 μm *(A)* and 100 μm *(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 ± 4.5	134.3 ± 16.9	226.0 ± 25.1	868.7 ± 296.5
Cells rolling in the blood vessel (cells/mm^2/30 min)	14.7 ± 1.5	33.3 ± 3.1	44.7 ± 4.5	2.7 ± 0.6
Cells accumulated in the field (cells/mm^2/30 min)	7.0 ± 1.5	15.3 ± 1.5	24.3 ± 2.5	1.3 ± 0.6
Cells egressed from the field (cells/mm^2/30 min)	7.0 ± 1.0	8.0 ± 1.5	8.3 ± 1.6	0.7 ± 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.

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 μg 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 T_H2-cell migration in the lung. Thus, T_H2 focus formation appears to be critical in the development of allergic airway inflammation in this animal model.

Eosinophilic infiltration and GFP^+ T_H2-cell infiltration into the lung in a mouse model of asthma

Previous studies in animal models suggest a T_H2 paradigm for allergic diseases, with an increased activation of T_H2 cells that produce T_H2 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 T_H2 cells observed in the current study and accumulation of eosinophils may be coincidental because our results showed

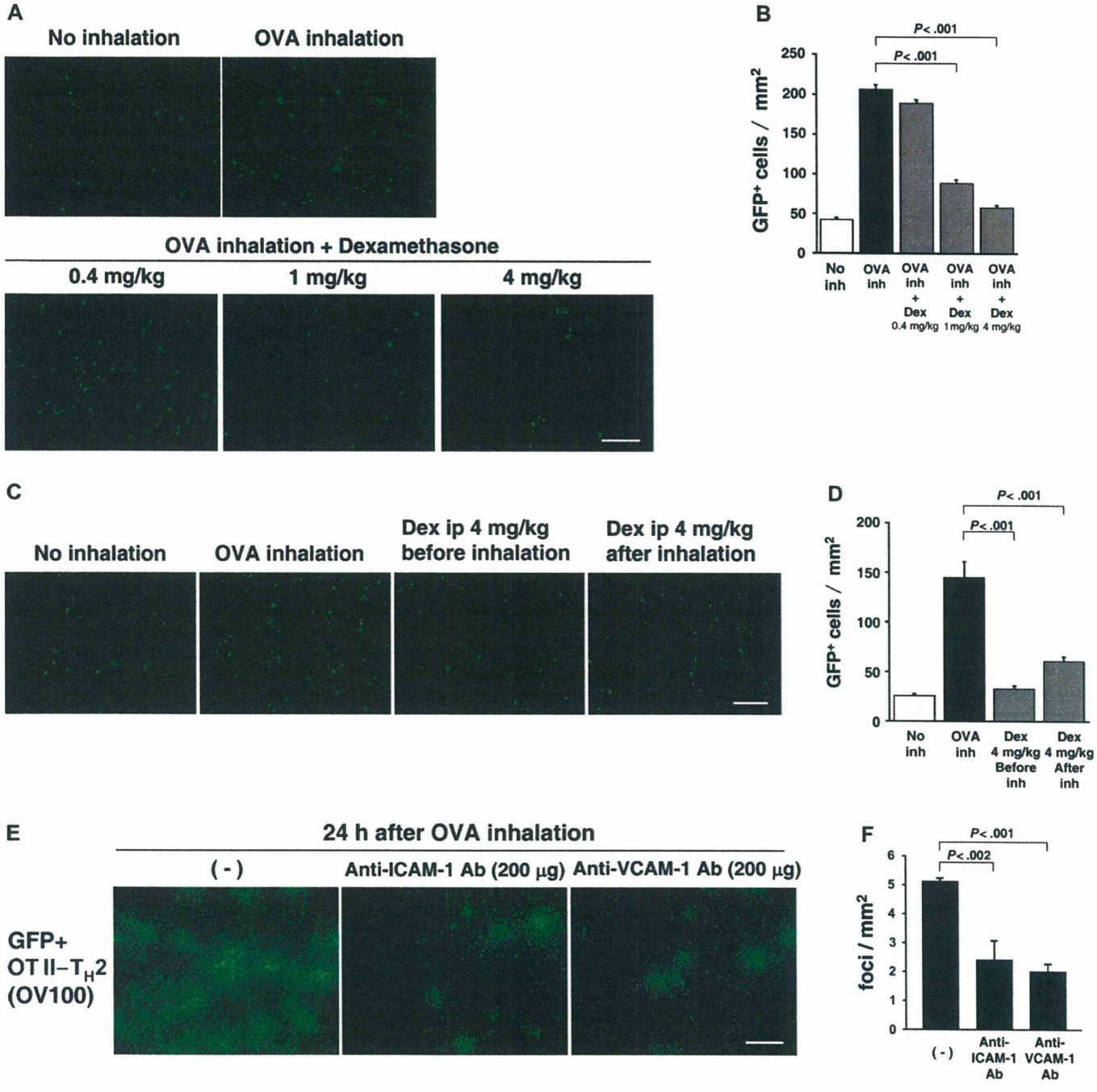

FIG 3. Effects of dexamethasone, anti–ICAM-1, and anti–VCAM-1 treatment on CD4 T-cell accumulation in the lung after ovalbumin (OVA) inhalation. **A,** Allergic airway inflammation was induced as in Fig 1, *A*. Three different doses of dexamethasone (DEX) were injected intraperitoneally 1 hour before 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.

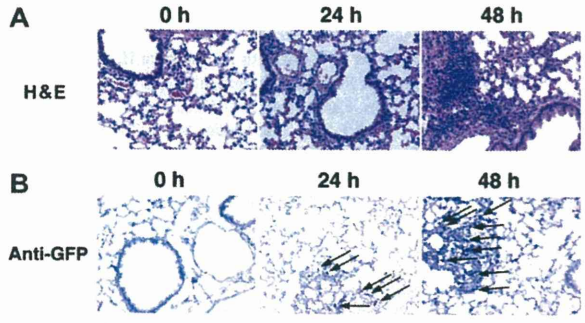

FIG 4. Eosinophilic infiltration and GFP$^+$ T_H2-cell infiltration into the lung in a mouse model of asthma. **A,** The lung specimens were fixed at indicated time point after ovalbumin inhalation and stained with H&E. A representative H&E staining pattern in each group is shown. Magnification ×200. **B,** Representative immunohistochemical staining for GFP is shown. Magnification ×200. *Arrows* indicate some of the representative GFP-positive cells. The results are representative of 3 experiments.

that antigen-specific T_H2 cells accumulated and formed foci in the lung after allergen inhalation. GFP$^+$ OT I–T_H2 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–T_H2 cells were monitored by immunohistochemistry analysis with an anti-GFP antibody. We observed GFP$^+$ transferred T_H2 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 T_H2 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 al[29] 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 T_H2 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 T_H2-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 T_H2 cells play an important role in migration and focus formation. Moreover, T_H2-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' group[33] 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 T_H2 cells expressing GFP from GFP Tg mice[16] 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 T_H2 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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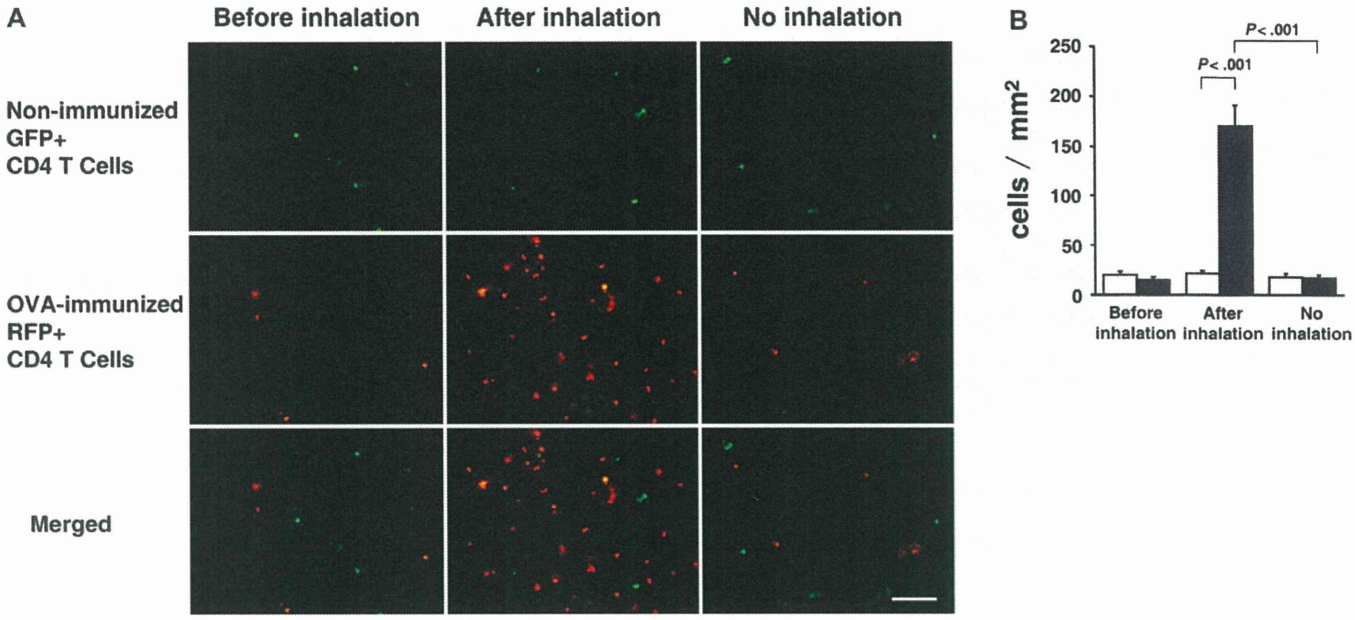

FIG E1. Color -coded fluorescence imaging of CD4 T-cell infiltration into the lung during ovalbumin (OVA)-induced allergic asthma. **A,** Color-coded images of CD4 T cells with the opposite color to the experiment shown in Fig 1, *B*. Splenic CD4 T cells from OVA-primed RFP Tg and nonprimed GFP Tg mice 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 μm. **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.