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IL-4 Induces Differentiation and Expansion of Th2 Cytokine-Producing Eosinophils[1]

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Innate effector cells that produce Th2-type cytokines are critical in Th2 cell-mediated immune responses. However, it is not known how these cells acquire the ability to produce Th2 cytokines. IL-4 is a potent inducer that directs differentiation of naive CD4$^+$ T cells into CD4$^+$ Th2 effector cells. To determine whether IL-4 can induce differentiation and expansion of Th2 cytokine-producing innate cells, we used mice whose *il-4* gene was replaced by a knock-in green fluorescence protein (*gfp*) gene. We found that, directly ex vivo, IL-4 increased the number of GFP$^+$ cells in the airway and the lung tissue in an Ag-specific manner. The majority of GFP$^+$ cells were eosinophils, suggesting that IL-4 plays a pivotal role in expanding IL-4-producing eosinophils in vivo. IL-4-producing eosinophils showed some unique features compared with IL-4-producing CD4$^+$ T cells. They exhibited biallelic expression of the *il-4* gene when stimulated and were more dominant IL-4- and IL-5-producing cells. Furthermore, we show that IL-4 drove bone marrow progenitor cells to differentiate into Th2 cytokine-producing eosinophils in vitro. These results strongly suggest IL-4 is a potent factor in directing bone marrow progenitor cells to differentiate into Th2 cytokine-producing eosinophils. ***The Journal of Immunology*****, 2004, 172: 2059–2066.**

The CD4$^+$ Th2 effector cells play important roles in generating protective immunity against parasitic infection or in causing tissue damage in allergy and asthma (1–4). IL-4 has been shown to be essential in generating CD4$^+$ Th2 effector cells both in vivo and in vitro (5–7). IL-4 mediates its functions by binding to the IL-4Rα chain and common γ-chain. The ligand receptor interaction results in activation of Janus kinase (JAK)[4] 1 and JAK3 (8, 9). Activated JAK1 and JAK3 then phosphorylate tyrosine residues located in the cytoplasmic domain of the IL-4Rα chain. The phosphorylated tyrosine residues then serve as docking sites to recruit STAT6 (10), which has been shown to enhance the expression of two key Th2-specific transcription factors, GATA3 and c-maf (11, 12). When IL-4Rα chain, STAT6, GATA3, or c-maf expression is disrupted, IL-4 fails to induce typical Th2 responses (13–16).

Innate effector cells that produce Th2-type cytokines are also critical in Th2 cells-mediated immune responses. In parasitic infection models, it has been demonstrated that eosinophils produce IL-4 that is imperative in generating protective immunity against parasitic infection (17, 18). In asthmatic patients, eosinophils have been shown to produce IL-4, IL-5, and IL-13 (19–22). It has also been reported that mast cells and basophils possess the capacity to produce Th2 cytokines (23–25). However, it is unknown how these innate effector cells acquire the capacity to produce Th2 cytokines. The IL-4R is widely expressed on many types of cells. It is possible that IL-4 can direct bone marrow progenitor cells into IL-4-producing innate cells that could function as innate effector cells of Th2 type.

In this report, by using IL-4/green fluorescence protein (GFP) reporter mice that either have or have no functional IL-4, we demonstrate that IL-4 can induce differentiation of Th2 cytokine-producing eosinophils both in vivo and in vitro.

Materials and Methods

Mice and immunization protocol

Dr. W. E. Paul's laboratory (Bethesda, MD) generated the IL-4/GFP reporter mice by replacing the first exon and 178 nt of the first intron of the *il-4* gene with gene-encoding enhanced GFP (26). A breeding pair of the IL-4/GFP reporter mice (129 backcrossed to C57BL/6 mice for 12 generations) was provided to us by Dr. Paul. These mice were fed a protein-free diet (Harlan 7012; Harlan Laboratories, Haslett, MI). Handling of animals complied with the animal protocol approved by the Loyola University Medical School Institutional Animal Care and Use Committee. In a typical immunization protocol, IL-4/GFP heterozygous (4GFP het) and GFP homozygous (4GFP hom) mice were given i.p. injection of 100 μg of chicken OVA, keyhole limpet hemocyanin (KLH), or PBS plus alum (Imject Alum; Pierce, Rockford, IL) on days 0 and 14. Seven days after the second i.p. injection, mice were anesthetized briefly with halothane (Holocarbon, River Edge, NJ) and given an intranasal injection of 400 μg of OVA or KLH in PBS daily for 3 consecutive days. Bronchoalveolar lavage (BAL) fluids, blood samples, and lung tissues were collected for FACS analysis 48 h after the third intranasal challenge. Lung tissues were taken routinely for pathological analysis.

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[4] Abbreviations used in this paper: JAK, Janus kinase; GFP, green fluorescence protein; 4GFP hom, IL-4/GFP homozygous; 4GFP het, IL-4/GFP heterozygous; MBP, major basic protein; EPO, eosinophil peroxidase; BAL, bronchoalveolar lavage; KLH, keyhole limpet hemocyanin; lin, lineage; SCF, stem cell factor; RT, reverse transcription; cRPMI, complete RPMI 1640.

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Pathological analysis

Lung tissue sections were processed and stained with H&E and periodic acid-Schiff to visualize goblet cells. For quantitative analysis of eosinophils, cytospins of BAL were prepared and stained with May-Grünwald-Giemsa staining. A total of 300 randomly selected cells were counted for differential cell analysis. For goblet cell counting, we counted the number of goblet cells within the epithelium lining of bronchioles that were also randomly selected. All results were counted blindly by two trained individuals and verified by two pathologists (H.B.X., Duke University, Durham, NC and S.A., Loyola Medical School, Maywood, IL).

Th2 cell culture

Lymph node cells from 4GFP hom and 4GFP het mice were depleted of $CD8^+$ cells, $B220^+$ cells, and IA^{b+} cells by negative selection using magnetic beads. The purified $CD4^+$ cells were then centrifuged on a discontinuous 50, 60, and 70% Percoll gradient. Cells with a density of >70% were collected and used as naive $CD4^+$ T cells for priming as described previously (27). Th2 priming was conducted by culturing 10^6 naive $CD4^+$ T cells in the presence of 10^7 irradiated T-depleted spleen cells, anti-CD3 (2C11, 3 μg/ml), anti-CD28 (37.N.51.13 μg/ml), IL-2 (10 U/ml), anti-IL-12 Ab (R&D Systems, Minneapolis, MN), and IL-4 (5 ng/ml) for 5 days.

Methylcellulose culture

Bone marrow cells of 4GFP hom mice were depleted of cells that expressed CD4, CD8, B220, and class II molecules by negative selection using a magnetic bead method. The resultant cells were used as lineage-negative (lin^-) cells. We achieved >99% purity for preparations of lin^- cells as judged by fluorescence microscope or by FACS analysis. In some experiments, we eliminated cells that expressed even low levels of fluorescence in the lin^- cell preparations by FACS sorting to ensure that they were completely GFP^- cells. Then 2×10^5 lin^-GFP^- cells were seeded in a 1% methylcellulose culture (Methocult M3234; StemCell Technologies, Vancouver, BC, Canada). Stem cell factor (SCF; 25 ng/ml), IL-4 (5 ng/ml), IL-5 (20 ng/ml), or IL-13 (10 ng/ml; PeproTech, Rocky Hill, NJ) was added to the cultures alone or in combination with the other ILs. Cells were incubated for 9–10 days. Colonies were distinguished by morphology and scored. Typically, we observed five types of colonies. Type 1 colonies were small in size and composed of small cells. This type of colony contained several distinct clumps of small cells. Type 2 colonies were also small in size, but there were no distinct clusters of cells within the colonies. Type 3 colonies resembled the typical monocyte colonies; i.e., colony and cell size was large. Type 4 colony and cell sizes were generally larger than those observed in type 3 colonies; they resembled megakaryocyte colonies. Type 5 colonies resembled eosinophil colonies. Cells within the colonies appeared to pile up in several layers in the center. The same type of colonies were picked, pooled, and stained using H&E for morphologic analysis. GFP^+ cells were analyzed using either a fluorescence microscope or FACScan (BD Biosciences, Mountain View, CA).

Flow cytometric analysis, intracellular staining, and ELISA

BAL cells were collected as described previously (28). The airway was washed with 1 ml of PBS before lung tissues were cut into small pieces. The tissue pieces were subjected to collagenase treatment (0.5 mg/ml) for 1 h in a 37°C water bath. Single cells were collected and RBCs were lysed with 0.15 M NH_3Cl. Number of cells recovered was counted. Single cells in some cases were stained with allophycocyanin-labeled anti-CD4 Ab. Cells were analyzed for GFP expression by using a FACScan (BD Biosciences). Dead cells were excluded by propidium iodide staining.

For intracellular staining, combined (1×10^6) BAL cells and lung cells in 1 ml of complete RPMI 1640 (cRPMI) medium were not stimulated or stimulated with PMA (10 ng/ml) and ionomycin (1 μM) in the presence of 2 μM monensin (Calbiochem, La Jolla, CA). Six hours later, both the stimulated and unstimulated cells were treated with 20 μg/ml DNase I (Boehringer Mannheim, Mannheim, Germany) for 5 min at 37°C, washed with cold PBS, fixed with 4% paraformaldehyde for 5 min at 37°C, washed with buffer containing 0.1% saponin and 0.1% BSA, and stained with PE-labeled anti-IL-4 (11B11) mAb (BD PharMingen, San Diego, CA) and allophycocyanin-labeled anti-CD4 Ab (BD PharMingen) (29). Samples were analyzed using a FACScan.

For analyzing the percentage of $CD4^+GFP^+$ cells in $CD4^+$ cells, we set a gate on $CD4^+$ cells and determined the percentage of $CD4^+GFP^+$ cells in $CD4^+$ cells with an analysis region. For $CD4^+GFP^+$ cells in total, we obtained the numbers using an analysis region that was set on $CD4^+GFP^+$ cells. We calculated the absolute number of $CD4^+GFP^+$ cells per animal by multiplying the percentage of $CD4^+GFP^+$ cells in total with the total number of BAL or lung cells. We used similar methods for analyzing the percentage of $CD4^-GFP^+$ cells. We gated on the $CD4^-$ population and determined the percentage of $CD4^-GFP^+$ cells in $CD4^-$ cells with an analysis region. For $CD4^-GFP^+$ cells in total, we used an analysis region that was set on $CD4^-GFP^+$ cells. To calculate the absolute number of $CD4^-GFP^+$ cells per animal, we multiplied the percentage of $CD4^-GFP^+$ cells in total with the total number of BAL or lung cells. Data from single animals were used to calculate mean ± SEM. Differences between treatment groups were analyzed by an unpaired Student's *t* test.

IL-5 protein and serum IgE were measured using commercial ELISA detection kits (BD PharMingen). The detection limits for IL-5 and IgE were 4 pg/ml and 2 ng/ml, respectively.

Electronic sorting

GFP^+ cells were electronically sorted by using a BD FACStar^{Plus} instrument. More than 99% of the sorted cells were GFP^+ when examined under a fluorescence microscope. For morphological analysis, GFP^+ cells were collected in a test tube and spun onto a glass slide for H&E or May-Grünwald-Giesma staining. Images were captured at ×60 magnification with a Nikon E600 microscope (Tokyo, Japan) equipped with a digital camera.

RT-PCR

$CD4^+GFP^+$ or $CD4^-GFP^+$ cells were sorted directly onto 10 μl of reverse transcription (RT) reaction mix containing 40 U Moloney leukemia virus-RT (Promega, Madison, WI), 100 μg/ml BSA, 1 ng/μl oligo$(dT)_{12–18}$, 250 μM each dNTP, 1 mM DTT, 100 μg/ml yeast tRNA, and 1% Triton X-100. cDNA was synthesized at 42°C for 60 min. Nested PCR was performed using designed primers. IL-5 primer sequences are as follows: first set forward, 5′-GCACTTGAGTGTTCTGACTC-3′; IL-5 reverse, 5′-GTTCCATCTCCAGCACTTCATG-3′; second set forward, 5′-CAATGAGACGATGAGGCTTC-3′; reverse, 5′-CCACTCTGTACTCATCACAC-3′. The IL-13 primer sequences are as follows: first set forward, 5′-ACAGCTCCCTGGTTCTCTCA-3′; reverse, 5′-CGTGGCGAAACAGTTGCTTTGTG-3′; second set: forward, 5′-TGCCAAGATCTGTGTCTCTC-3′; reverse, 5′-CAGTTGCTTTGTGTAGCTGAGC-3′.

The first round of PCR consisted of 40 cycles at 95°C for 20 s, 55°C for 30 s, and 72°C for 80 s, followed by 7 min at 72°C. One microliter of the first PCR product was used for the nested PCR template. The nested PCR consisted of 30 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 65 s, followed by 7 min at 72°C. Samples were separated on a 2% agarose gel. For eosinophil marker analysis, $2–20 \times 10^3$ $CD4^-GFP^+$ or $CD4^+GFP^+$ cells were sorted into a test tube containing 1 ml of medium. Cells were spun and collected. mRNA was prepared with a MicroPoly(A) Pure kit (Ambion, Austin, TX) according to the manufacturer's instructions. cDNA was synthesized with Moloney leukemia virus-RT (Promega). PCR was subsequently performed with the following primer pairs: eosinophil peroxidase (EPO) forward, 5′-CCTTTTGACAACCTGCATGA-3′ and reverse, 5′-CCCAGATGTCAATGTTGTCG-3′; major basic protein (MBP) forward, 5′-GGAGCGTCTGCTCTTCATCT-3′and reverse, 5′-ACTTCCATCAACCCATCGAA-3′; and CCR3, forward, 5′-TCCTGCCTCCACTGTACTCC-3′ and reverse, 5′-CGTGCTGTGAAAAGCAGAAA-3′ (30).

Results

4GFP het mice, but not 4GFP hom mice, develop features of allergic airway inflammation

Because we used the IL-4/GFP reporter mice that possess one copy of the IL-4 and one copy of the GFP gene (4GFP het) and mice that have two copies of the GFP gene (4GFP hom) to determine the role of IL-4 in allergic airway inflammation in vivo, we needed to rule out the possibility that the IL-4/GFP reporter mice, only exon 1 of the *il-4* gene was replaced by GFP, make a functional chimeric GFP/IL-4 fusion protein. We found that $CD4^+$ T cells from 4GFP hom mice primed under Th2-inducing conditions did not produce IL-4 protein by ELISA (Fig. 1*A*). To evaluate to what degree GFP expression report IL-4 protein expression, we primed naive $CD4^+$ T cells from 4GFP het mice with various amounts of IL-4 for 5 days. We measured the amount of IL-4 protein and GFP produced by the resultant cells. We plotted the percentage of GFP^+ cells against amounts of IL-4for each priming dose. We found a perfect correlation ($R = 1.00$) between percentage of GFP^+ cells and amounts of IL-4 protein produced (Fig. 1*B*). This curve could also

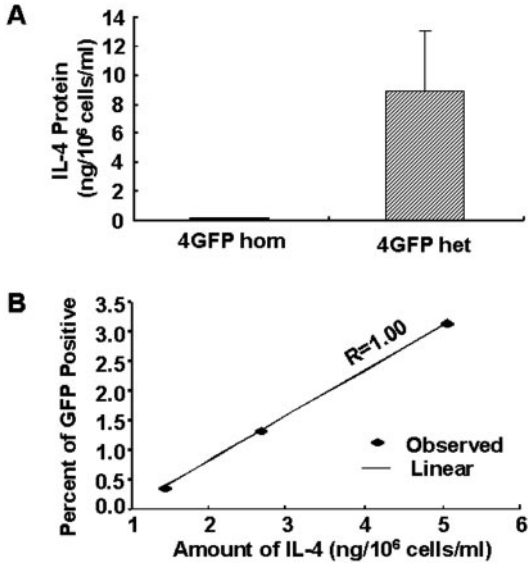

FIGURE 1. Th2-inducing condition-primed $CD4^+$ T cells from 4GFP hom mice fail to produce detectable IL-4. *A*, Naive $CD4^+$ T cells were prepared from lymph nodes of 4GFP hom and 4GFP het mice and primed in the presence of IL-4 (5 ng/ml) and anti-IL-12 (10 μg/ml) for 5 days. Cells were then washed and restimulated with PMA and ionomycin at a concentration of 1×10^6 cells in 1 ml of complete medium overnight. IL-4 proteins in the supernatants were measured by ELISA. *B*, Quantification of GFP expression in relative to IL-4 protein. Naive $CD4^+$ T cells were prepared from lymph nodes of 4GFP het mice and primed in the presence of IL-4 (0.1, 0.3, and 0.9 ng/ml) and anti-IL-12 (10 μg/ml) for 5 days. Cells were then washed and restimulated with PMA and ionomycin at a concentration of 1×10^6 cells in 1 ml of complete medium overnight. Supernatants were collected and used for IL-4 protein measurement by ELISA. Cells were analyzed for GFP expression by FACS. Correlation between percentage of GFP^+ cells and amounts of IL-4 produced was analyzed with linear regression.

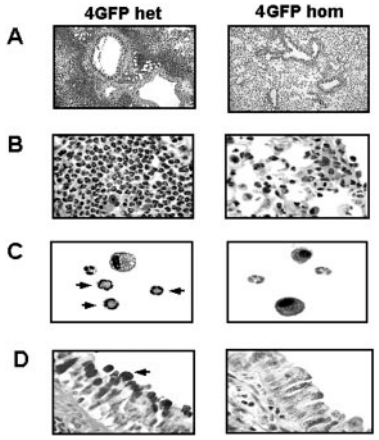

FIGURE 2. IL-4 plays critical roles in developing features of allergic airway inflammation. 4GFP het and hom mice were given i.p. and intranasal injections of PBS or 100 μg of OVA plus alum on days 0 and 14. On days 21, 22, and 23, sensitized mice were given intranasal injections of PBS or OVA (400 μg). Forty-eight hours after the last intranasal challenge, lung tissue sections were prepared and stained with H&E or periodic acid-Schiff. *A*, Parabronchiole cell infiltration. Photographs were taken at a magnification of $\times$10. *B*, Higher magnification of lymphocyte and eosinophil filtration in the lung parenchyma parabronchial and paravascular areas. *C*, Eosinophilia in the BAL (arrows). *D*, Goblet cells (arrow) in the epithelial lining of the bronchiole. From *B* through *D*, photographs were taken at a magnification of $\times$40. These results are representative of five independent experiments.

be used for estimating amounts of GFP equivalent to amounts of IL-4 protein.

To examine whether a single copy of IL-4 in 4GFP het mice can induce features of allergic airway inflammation, we sensitized and challenged 4GFP hom and 4GFP het mice with OVA. We found that 4GFP het mice developed typical features of allergic airway inflammation, whereas 4GFP hom mice did not. Three parameters including eosinophil infiltration in the BAL fluid, goblet cell hyperplasia, and total serum IgE were used to measure the degree of allergic airway inflammation. The parabronchial and paravascular areas were infiltrated with eosinophils and lymphocytes in the lung of 4GFP het mice but not in that of 4GFP hom mice (Fig. 2, *A* and *B*). The percentage of eosinophils in BAL cells in 4GFP het mice increased compared with that of 4GFP hom mice (Fig. 2*C* and Table I). The number of goblet cells within the epithelial lining of the bronchioles of 4GFP het mice was also elevated greatly in 4GFP het mice compared with that of 4GFP hom mice (Fig. 2*D* and Table I). The total serum IgE of 4GFP het mice was significantly higher than that of 4GFP hom mice (Table I). 4GFP het mice developed comparable features of allergic inflammation to C57BL/6 control mice (Table I). Thus, a single copy of IL-4 functions equally well as two copies of IL-4 (wild type) in developing allergic airway inflammation.

Accumulation of GFP^+ cells into the lung and airway was regulated by Ag-specific responses and was largely IL-4 dependent

We first measured the percentage of GFP-expressing cells by FACS in the combined BAL and lung cells of 4GFP hom and het mice that received various treatments. GFP^+ cells were defined in Fig. 3*A*. The percentage of GFP^+ cells in the lung and airway of 4GFP het mice increased markedly compared with that of 4GFP hom mice and to that of 4GFP het mice receiving KLH or PBS treatments (Fig. 3*B*). Albeit to the less degree, the percentage of GFP^+ cells in the lung and airway of 4GFP hom mice given OVA also increased significantly compared with that of 4GFP hom mice given PBS or KLH (Fig. 3*B*). These data suggest that the accumulation of GFP^+ cells into the lung and airway was regulated by Ag-specific responses and was largely IL-4 dependent. Moreover, the experimental results also led us to conclude that IL-4-producing cells can differentiate and can be recruited into the lung and the airway in response to Ag stimulation alone in the absence of IL-4. However, IL-4 is critical in expanding the number of IL-4-producing cells to sufficient numbers that may be required to cause airway inflammation.

It is known that $CD4^+$ T cells can produce IL-4. We used CD4 as a marker to divide GFP^+ cells into $CD4^+GFP^+$ and $CD4^-GFP^+$ cell population (Fig. 4*A*). $CD4^-GFP^+$ cells were then isolated electronically from pooled BAL and lung cells prepared from 4GFP het mice for morphological analysis. We achieved >99% purity. Nearly all $CD4^-GFP^+$ cells (93%) were eosinophils (Fig. 4*B*), 4% were monocytes, and 2.6% were blast-like cells by H&E staining; only one mast cell of >1000 $CD4^-GFP^+$ cells was detected (result not shown). In some cells that had bilobed and donut-shaped nuclei, we did not observe the cytoplasm to be full of eosinophilic granules, presumably due to degranulation in vivo. Nevertheless, they were considered as eosinophils based on their morphology. Furthermore, isolated $CD4^-GFP^+$ cells expressed mRNA for CCR3, MBP, and EPO, whereas isolated $CD4^+GFP^+$ cells did not express MBP or EPO although they expressed lower levels of CCR3 mRNA (Fig. 4*C*). These results suggest that the majority of $CD4^-GFP^+$ cells were eosinophils.

Table I. *Features of allergic inflammation*

Strain	Serum IgE[a] (ng/ml)		Eosinophils in BAL[b] (%)	Goblet Cells[c] (/mm^2)
	PBS	OVA	OVA	OVA
4GFP hom	Undectable	20.8 ± 5.2	5.0 ± 0.7	1.0 ± 0.5
4GFP het	99.9 ± 40.7	1071.7 ± 160.6	51.0 ± 6.5	47.0 ± 5.2
C57BL/6 wild type	38.4 ± 2.3	1728.5 ± 585.4	39.0 ± 2.5	31.0 ± 1.7

[a] Mean ± SEM derived from five independent experiments. The number of animal used for each group was between 3 and 10.

[b] Eosinophils were counted from BAL cell cytospin preparations. A total of 300 randomly selected cells per slide were counted. The data were presented as percentage of eosinophils in total number of cells counted.

[c] For goblet cell counting, three random sections were selected. For each section, we randomly selected three areas that surrounded bronchial or blood vessels. Data are presented as the number of cells per square millimeter.

The number of IL-4-producing eosinophils undergoes remarkable expansion in the lung and the airway of 4GFP het mice

To determine the role of IL-4 in expanding the number of IL-4-producing CD4$^+$ and eosinophils, we measured the percentage of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells in the BAL and lung separately. The percentage as well as total number of CD4$^+$GFP$^+$ cells in the lung of 4GFP het mice increased 3- to 3.5-fold compared with that of 4GFP hom mice. The increase was even more pronounced in the BAL. The percentage of CD4$^+$GFP$^+$ cells increased 4.5-fold, while the total number of CD4$^+$GFP$^+$ cells increased 11.5-fold (Table II). The role of IL-4 in the expansion of IL-4-producing eosinophils (CD4$^-$) was equally remarkable. The percentage of cells as well as the total number of CD4$^-$GFP$^+$ cells increased 3.8- to 4.5-fold and 3.4- to 7-fold in the lung and the BAL, respectively (Table II). These results indicate that IL-4 plays a critical role in differentiation and expansion of both IL-4-producing CD4$^+$ T cells and eosinophils in vivo.

To compare the relative contribution of CD4$^+$ T cells and eosinophils to the IL-4 production, we used the same analysis gate to analyze the percentage of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells in total lung and BAL cells obtained from 4GFP het mice. We found that the percentage of CD4$^-$GFP$^+$ cells was 2-fold higher in the lung and 4-fold higher in the BAL than that of CD4$^+$GFP$^+$ cells (Table II). The total number of CD4$^-$GFP$^+$ cells in the lung and the BAL approximately was 2-fold greater than that of

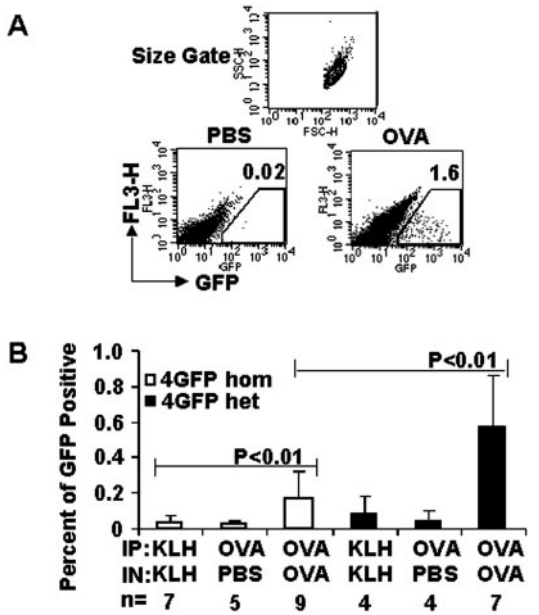

FIGURE 3. GFP expression is regulated in an Ag-specific manner. 4GFP hom and het mice were given i.p. and intranasal injections of OVA, KLH, or PBS. Lung cells and BAL cells from the same individual mouse were prepared and pooled 48 h after the third intranasal challenge. *A*, GFP$^+$ cells were analyzed by FACS. GFP$^+$ cells were defined in the region as shown. The size gate used for data analysis is also shown. *B*, Percent GFP positive represents the percentage of GFP$^+$ cells in combined total BAL and lung cells. Error bars represent SD. These data were calculated from nine independent experiments. The number of individual animals used is indicated.

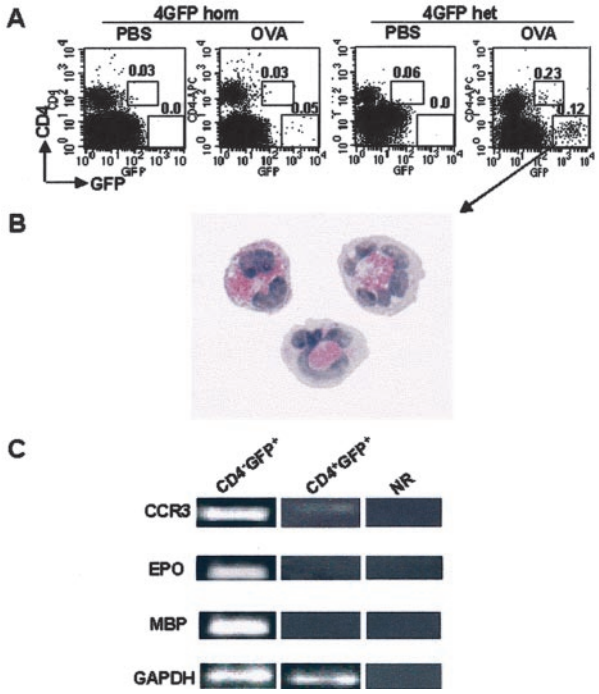

FIGURE 4. Most CD4$^-$GFP$^+$ cells are eosinophils. *A*, BAL and lung cells (1×10^6) prepared and combined from 4GFP hom and het mice 48 h after the last intranasal challenge. They were stained with allophycocyanin-labeled anti-CD4 Ab. FACS plots are representative of a typical experiment. CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells were defined in the regions as shown according to PBS control. *B*, CD4$^-$GFP$^+$ cells were isolated by electronic sorting from lung cells of 4GFP het mice 48 h after the third intranasal injection. The sorted cells were spun onto glass slides and then stained with H&E. Photographs were taken at a magnification of ×60. The data represent three independent experiments. *C*, CD4$^-$GFP$^+$ cells and CD4$^+$GFP$^+$ cells (3000 each) were isolated by FACS sorting and used for mRNA preparations. PCR was performed using primers designed for CCR3, MBP, EPO, and GAPDH. NR, No RT control.

Table II. *Expansion of $CD4^+$ GFP^+ and $CD4^-GFP^+$ cells*[a]

	Lung					
	$CD4^+$ GFP^+			$CD4^-$ GFP^+		
Strain	% in $CD4^+$	% In total	No. per mouse $\times 10^3$	% in $CD4^-$	% in Total	No. per mouse $\times 10^3$
4GFP hom	0.83 ± 0.17	0.07 ± 0.02	5.63 ± 1.70	0.13 ± 0.05	0.11 ± 0.04	10.22 ± 4.50
4GFP het	1.64 ± 0.32	0.22 ± 0.07	19.48 ± 5.90	0.56 ± 0.16	0.42 ± 0.11	46.37 ± 16.90
	BAL					
	$CD4^+$ GFP^+			$CD4^-$ GFP^+		
Strain	% in $CD4^+$	% in Total	No. per mouse $\times 10^3$	% in $CD4^-$	% in Total	No. per mouse $\times 10^3$
4GFP hom	0.76 ± 0.33	0.08 ± 0.03	0.74 ± 0.30	0.57 ± 0.32	0.42 ± 0.23	2.24 ± 1.10
4GFP het	4.02 ± 1.21	0.36 ± 0.12	8.53 ± 3.80	1.92 ± 0.62	1.44 ± 0.46	15.89 ± 5.20

[a] The number of animals used for each group was between 7 and 10. Data are represented as mean ± SEM. Significant differences (Student's *t* test) are observed: in the absolute numbers of $CD4^+$ GFP^+ cells between 4GFP het and 4GFP hom mice either in lung ($p = 0.0236$) or BAL ($p = 0.0381$) and in the absolute numbers of $CD4^-$ GFP^+ cells between 4GFP het and 4GFP hom mice either in lung ($p = 0.0323$) or BAL ($p = 0.0178$).

$CD4^+GFP^+$ cells (Table II). These data suggest that eosinophils were more dominant IL-4-producing cells.

Allelic expression of IL-4 is regulated differently in eosinophils than in $CD4^+$ T cells

To determine whether IL-4-producing eosinophils are different from $CD4^+$ Th2 cells in their ability to regulate *il-4* gene expression, we analyzed expression of the *il-4* gene and the *gfp* reporter gene in $CD4^+$ T cells and $CD4^-$ cells. BAL and lung cells were prepared from 4GFP het mice either directly ex vivo or after a short-term in vitro stimulation with PMA and ionomycin. We found that, directly ex vivo, 0.3% of $CD4^-$ cells expressed the IL-4 allele and 0.03% of $CD4^-$ cells expressed the gfp allele. None of them expressed both alleles. However, when stimulated with PMA and ionomycin, the percentage of $CD4^-$ cells expressing both alleles increased dramatically to 1.1%; the percentage of $CD4^-$ cells expressing IL-4 increased to 0.8%; and the percentage of $CD4^-$ cells expressing GFP increased to 0.3% (Fig. 5). Compared with $CD4^-$ cells, no detectable $CD4^+$ T cells expressed IL-4 although they expressed GFP (0.3%) directly ex vivo. None of the $CD4^+$ T cells expressed both IL-4 and GFP even when they were stimulated. The percentage of $CD4^+$ T cells expressing IL-4 increased to 2.3% and the percentage of $CD4^+$ T cells expressing GFP increased to 0.6% when stimulated (Fig. 5). These data led us to conclude that eosinophils more readily express both alleles when they encounter strong stimuli and thus may produce more IL-4 under these conditions. In addition, because of monoallelic expression of the *il-4* allele and *gfp* allele (i.e., $CD4^+GFP^+$ cells or $CD4^-GFP^+$ cells when they were not stimulated in vitro do not express IL-4), we interpret the results using the GFP reporter system as an indirect measurement of IL-4-producing capacity.

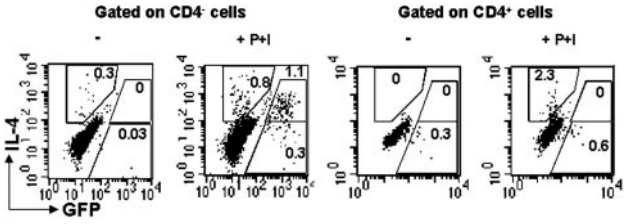

FIGURE 5. Allelic expression of IL-4 and GFP in $CD4^-$ cells differ from $CD4^+$ cells. 4GFP het mice were given i.p. and intranasal injections of OVA. Forty-eight hours after the last intranasal challenge, pooled BAL and lung cells (1×10^6 in 1 ml of cRPMI medium) were not stimulated or stimulated with PMA and ionomycin (P + I) in the presence of monensin for 6 h. Then the cells were stained with allophycocyanin-labeled anti-CD4 and PE-labeled anti-IL-4 Ab by using an intracellular staining method. A FACS analysis gate was set on $CD4^+$ (gated on $CD4^+$ cells) or $CD4^-$ populations (gated on $CD4^-$ cells). The numbers indicate the percentage of GFP^+ cells in $CD4^+$ cells or in $CD4^-$ cells.

GFP^+ eosinophils produce more IL-5 than do GFP^+CD4^+ T cells

To examine whether $CD4^-GFP^+$ cells can produce more of other types of Th2 cytokine, we measured IL-5 protein produced by $CD4^+GFP^+$ cells and by $CD4^-GFP^+$ cells. Pooled BAL and lung cells were prepared from 4GFP het mice that were given the third intranasal injection of OVA 48 h before the assay. $CD4^-GFP^+$ cells were electronically sorted. The sorted cells were stimulated with PMA and ionomycin overnight. We detected 3.2-fold more IL-5 protein produced by $CD4^-GFP^+$ cells than that by $CD4^+GFP^+$ cells (Fig. 6). Thus, eosinophils appear to be more dominant IL-5-producing cells in the late-phase response of allergic airway inflammation.

IL-4 directs bone marrow progenitors to differentiate into Th2 cytokine-producing eosinophils

To determine whether IL-4 can induce bone marrow progenitor cells to differentiate into Th2 cytokine-producing eosinophils, we

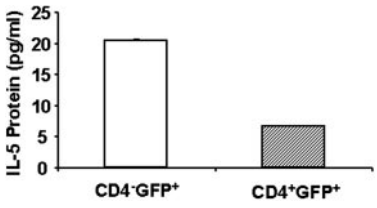

FIGURE 6. $CD4^-GFP^+$ cells produce more IL-5 protein. BAL and lung cells were prepared and combined from four 4GFP het mice that were sensitized and given the third intranasal injection of OVA 48 h before the assay. Combined BAL and lung cells were stained with allophycocyanin-labeled anti-CD4 Ab. $CD4^-GFP^+$ cells and $CD4^+GFP^+$ cells (3000 each) were sorted electronically into a test tube containing complete medium. The sorted cells in 50 μl of cRMPI were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) overnight. Supernatants were collected and used for IL-5 protein measurement by ELISA. Error bars represent SEM derived from duplicate measurements (0.28 for $CD4^-GFP^+$ cells and 0.03 for $CD4^+GFP^+$ cells). Significant differences in IL-5 production were found between $CD4^-GFP^+$ cells and $CD4^+GFP^+$ cells. $p = 0.004$, unpaired Student's *t* test).

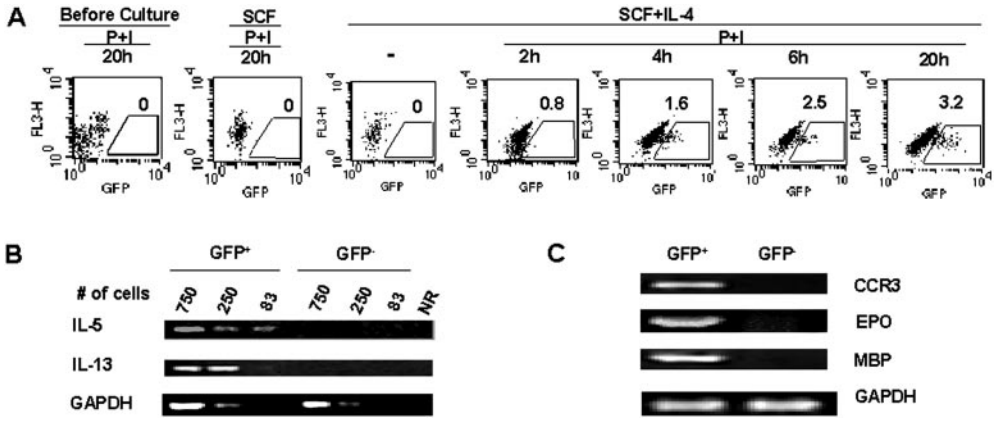

FIGURE 7. IL-4 primes lin$^-$GFP$^-$ bone marrow progenitor cells to become IL-4-producing eosinophils. *A*, lin$^-$GFP$^-$ cells (5×10^4) prepared from 4GFP hom mice were not cultured or cultured in 1% methylcellulose for 9 days with SCF (25 ng/ml) or SCF and IL-4 (5 ng/ml). Colonies were pooled and not stimulated (−) or stimulated with PMA and ionomycin (P + I) for the time indicated. lin$^-$GFP$^-$ preparation before culture was also stimulated with PMA and ionomycin overnight (before culture). GFP$^+$ cells were analyzed with FACS. *B*, Cells from SCF (25 ng/ml) plus IL-4 (5 ng/ml)-treated cultures were stimulated with PMA and ionomycin for 4 h. GFP$^-$ and GFP$^+$ cells were electronically sorted and used for mRNA and cDNA preparations. PCRs were performed with primers designed for amplification of IL-5, IL-13, and GAPDH. # of cells, Number of cells used per RT-PCR. Thirty-five cycles of PCR were performed. *C*, GFP$^-$ and GFP$^+$ cells were electronically sorted from SCF (25 ng/ml) plus IL-4 (5 ng/ml) cultures and used for mRNA and cDNA preparations. PCRs (35 cycles) were performed with primers designed for amplification of CCR3, EPO, MBP, and GAPDH.

established an in vitro culture system. We prepared lin$^-$GFP$^-$ bone marrow progenitor cells from unimmunized 4GFP hom mice and stimulated them with PMA and ionomycin overnight to test whether there are small percentages of lin$^-$GFP$^-$ progenitor cells already committed. lin$^-$GFP$^-$ progenitor cell preparation did not express GFP (Fig. 7*A*). This result suggests that our preparation of lin$^-$GFP$^-$ cells were uncommitted cells. Next, the purified lin$^-$GFP$^-$ cells were seeded onto 1% methylcellulose with addition of SCF alone or in combination with IL-4 for 9 days. After 9 days of culture, we found that most colonies in IL-4-treated cultures were small (type 1 or type 2 as described in *Materials and Methods*), containing 4–10% eosinophils, 8–28% monocytes, 46–74% neutrophils, and 8–12% blast-like cells. For FACS analysis, all colonies were pooled. The colony cells were not stimulated or stimulated with PMA and ionomycin for periods of time indicated (Fig. 7*A*). The colony cells did not express GFP without stimulation. When stimulated with PMA and ionomycin for 4 h, significant numbers of the colony cells expressed GFP. The number of GFP$^+$ cells was even higher after 20 h of stimulation (Fig. 7*A*). Sixty to 80% of colony cells recovered after 20 h of stimulation were viable. Like those CD4$^-$GFP$^+$ cells isolated from 4GFP het mice directly ex vivo, the GFP$^+$ cells derived in vitro also expressed IL-5 and IL-13 mRNA (Fig. 7*B*). They also expressed eosinophil-specific or enhanced MBP, EPO, and CCR3 mRNA (Fig. 7*C*). Morphologically, the in vitro-derived GFP$^+$ cells were typical eosinophils (data not shown). These results suggest that IL-4 can direct bone marrow progenitor cells to differentiate into Th2 cytokine-producing eosinophils.

Discussion

In this report, we sought to determine whether IL-4 could induce differentiation and expansion of Th2 cytokine-producing innate immune cells during OVA-induced allergic airway inflammation. By using IL-4/GFP reporter mice in which one or both alleles of the *il-4* gene has been replaced by a knock-in gfp gene, we demonstrated that IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils both in vivo and in vitro.

IL-4 has a rapid turnover rate. It is difficult to measure IL-4 expression without using a short-term in vitro stimulation. In this study, we took advantage of stable expression of GFP and measured GFP expression directly ex vivo without further in vitro stimulation to estimate IL-4 expression in vivo. Although the numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ appear to be small, they were measured directly ex vivo without giving them a short-term in vitro stimulation. In fact, when stimulated for 6 h, the numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells increased (Fig. 5). If cells obtained directly ex vivo were stimulated overnight, the numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells reported in our studies could have increased further. Most measurements of IL-4 production by cells obtained from mice were performed using a short-term in vitro stimulation (31, 32). However, using a short-term in vitro stimulation may overestimate the number of IL-4-producing cells. To more accurately interpret the meaning of using numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells as measurements, we developed a standard curve to correlate the percentage of GFP$^+$ cells to amounts of IL-4 being produced by the cells that were treated with identical conditions (Fig. 1*B*). Based on the curve, 1% of GFP$^+$ cells could produce ~2 ng/ml IL-4, which was equivalent to amounts of IL-4 produced by in vitro-primed CD4$^+$ Th2 cells that were restimulated with PMA and ionomycin overnight. More importantly, we correlate the numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells, measured directly ex vivo, with pathological changes. These findings together suggest that the numbers of CD4$^+$GFP$^+$ and CD4$^-$GFP$^+$ cells found in our studies may be sufficient to cause tissue damage.

Th2 cytokine-producing eosinophils exhibit several unique characters compared with CD4$^+$ Th2 cells. In CD4$^+$ T cells, our results demonstrated monoallelic IL-4 expression. These data are consistent with previous reports on monoallelic IL-4 expression by CD4$^+$ T cells cultured in vitro (26, 33, 34). IL-4-producing eosinophils, in contrast, more readily express both the IL-4 allele and GFP allele in response to strong stimuli. We observed that eosinophils were more dominant IL-4-, as measured by GFP expression, and IL-5-producing cells than CD4$^+$ Th2 cells. These data suggest that the epigenetic regulation of IL-4 allelic expression may be different between CD4$^+$ Th2 cells and Th2 cytokine-producing eosinophils. One caveat regarding the interpretation of these data is the use of PMA and ionomycin in our experiments. We consider

PMA and ionomycin stimulation as a means to measure IL-4-producing potential or capacity rather than the actual in vivo expression of IL-4.

Differentiation of IL-4-producing eosinophils is not entirely IL-4 dependent. We observed that, in the absence of IL-4, the percentage as well as the number of $CD4^+GFP^+$ and $CD4^-GFP^+$ cells increased significantly when the 4GFP hom mice were challenged with OVA, albeit to a much less degree compared with that of 4GFP het mice. These results suggest that initiation of a Th2-type inflammatory response may not require IL-4, as Ag stimulation alone is sufficient. Indeed, a number of groups have reported the existence of IL-4-independent IL-4-producing cells (18, 35–37).

However, successful expansion of the number of Th2-type effector cells may be the key in generating protective immunity or in causing tissue damage. Our study demonstrated that IL-4 is critical in expanding not only the number of $CD4^+$ Th2 cells, but also, even more remarkably, the number of Th2 cytokine-producing eosinophils. The importance of IL-4-producing eosinophils in protective immunity against infection with helminth has been recently established (18). Locksley and colleagues (18) reported that in a parasitic model, Th cells are not required for the generation of IL-4-producing eosinophils, but they are essential for expansion and maintenance of these cells. We have extended the finding in the allergic airway inflammation model and provide the cellular mechanism to explain the observation. Our new findings demonstrate that, in addition to the known functions of eosinophils in causing tissue damage by releasing inflammatory mediators from their granules, such as MBP, eosinophil cationic protein, EPO and eosinophil-derived neurotoxin (38), eosinophils can be the major source of Th2 cytokines in the late-phase response of allergic airway inflammation.

The in vivo source of IL-4 that directs progenitor cells to become Th2 cytokine-producing eosinophils remains to be determined. We think the following two scenarios are likely to occur. IL-4 produced by $CD4^+$ Th2 cells and $CD4^-$ cells after circulating out of the lung may prime bone marrow progenitor cells into Th2 cytokine-producing eosinophils in the bone marrow. Alternatively, circulating bone marrow progenitor cells can be recruited into the site of airway inflammation where they are exposed to IL-4 produced by $CD4^+$ Th2 cells or $CD4^-$ cells and differentiate into IL-4-producing eosinophils.

Our study raises the possibility that Th2 cytokine-producing eosinophils may regulate Th2 response in two aspects. First, in the absence of IL-4, eosinophils already possess the ability to produce IL-4, which may be used for directing naive $CD4^+$ T cells to differentiate into $CD4^+$ Th2 cells. Second, eosinophils or eosinophils progenitor cells are good responding cells to IL-4 stimulation. They differentiate and expand in the presence of IL-4 to become major effector cells of the Th2 type. These interplays may form a cellular circuit for the amplification of Th2 cytokines in allergic airway inflammation. Our study thus provides insights into the mechanism by which adaptive immune components may coordinate innate immune components to generate Th2-type immunity.

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