

# Limited Ability of Antigen-Specific Th1 Responses to Inhibit Th2 Cell Development In Vivo<sup>1</sup>

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Th1 and Th2 cells mutually antagonize each other's differentiation. Consequently, allergen-specific Th1 cells are believed to be able to suppress the development of Th2 cells and to prevent the development of atopic disorders. To determine whether a pre-existing Ag-specific Th1 response can affect the development of Th2 cells in vivo, we used an immunization model of Ag-pulsed murine dendritic cell (DC) transfer to induce distinct Th responses. When transferred into naive mice, Ag-pulsed CD8 $\alpha$ <sup>+</sup> DCs induced a Th1 response and the production of IgG2a, whereas CD8 $\alpha$ <sup>−</sup> DCs primed a Th2 response and the production of IgE. In the presence of a pre-existing Ag-specific Th2 environment due to Ag-pulsed CD8 $\alpha$ <sup>−</sup> DC transfer, CD8 $\alpha$ <sup>+</sup> DCs failed to prime Th1 cells. In contrast, CD8 $\alpha$ <sup>−</sup> DCs could prime a Th2 response in the presence of a pre-existing Ag-specific Th1 environment. Moreover, exogenous IL-4 abolished the Th1-inducing potential of CD8 $\alpha$ <sup>+</sup> DCs in vitro, but the addition of IFN- $\gamma$  did not effectively inhibit the potential of CD8 $\alpha$ <sup>−</sup> DCs to prime IL-4-producing cells. Thus, Th1 and Th2 cells differ in their potential to inhibit the development of the other. This suggests that the early induction of allergen-specific Th1 cells before allergy sensitization will not prevent the development of atopic disorders. *The Journal of Immunology*, 2005, 174: 1325–1331.

Immune responses to Ags are heterogeneous with respect to the cytokines produced by Ag-specific Th lymphocytes and the class of Abs secreted by B cells. Th1 cells secrete IL-2 and IFN- $\gamma$  and promote cellular immunity and the production of IgG2a. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and thereby induce the production of IgE and promote eosinophil-mediated inflammation (1, 2). Once the deviation toward either a Th1 or a Th2 response begins, additional polarization is promoted by the cytokines that are produced by Th cells. This is because the Th1 cytokine IFN- $\gamma$  suppresses Th2 cell proliferation and promotes Th1 cell differentiation, whereas the Th2 cytokines promote additional Th2 differentiation and inhibit the development of Th1 cells (3, 4). Th1 and Th2 responses are thus considered to be mutually exclusive and reciprocally regulated.

Atopy is an immune disorder characterized by hypersensitivity to common, usually innocuous environmental Ags, and its increasing incidence is a major concern of Western and developed societies. Atopic disorders have in common the elevation of allergen-specific IgE Abs and chronic inflammation typified by the predominant recruitment of eosinophils. These characteristics suggest that an inappropriate Th2-biased immune response to allergens is central to the pathogenesis of atopic disorders (2, 5). This together with the mutual antagonism between Th1 and Th2 cells

have led to the hypothesis that the increase in the incidence of atopic disorders is linked to a decrease in the prevalence of infections early in life that induce Th1 responses. It is believed that these early infections may remold the normally Th2-biased neonatal immune system into a Th1-biased one that is less susceptible to allergens (6). Supporting this idea are several epidemiological studies that show an inverse relationship between the development of atopy and the incidence of early infections (7, 8). In addition, atopic infants exhibit a slower acquisition of an IFN- $\gamma$ -producing capacity and a persistent Th2 phenotype compared with nonatopic infants (9). It has also been suggested that the latter are protected from allergic disorders because they have allergen-specific Th1 responses. IFN- $\gamma$  that is produced by these Th1 cells during an encounter with allergens is believed to be sufficient to suppress the development of allergen-specific Th2 cells (10).

However, there are observations that do not conform to the view that the stimulation of Th1 responses, at least in early childhood, counteracts the development of atopic disorders. First, the incidence of Th1-mediated autoimmune disorders is increasing in parallel with that of Th2-mediated allergic pathologies (11–13). Second, there is a lower prevalence of atopy among persons infected with helminths, which induce strong Th2 responses (14). Third, passive transfer of Ag-specific Th1 cells could not prevent the development of Th2 cells in a murine model of asthma (15).

These contradictions prompted us to investigate the effect of a pre-existing Ag-specific Th1 response on the development of Th2 response in vivo. Several studies have previously shown that the presence of Th1-inducing stimuli and Th1 cytokines inhibit the development of allergen-specific Th2 cells (10, 16). However, these studies were performed using exogenous cytokines and artificial adjuvants; hence, their observations may not reflect what really happens under physiological conditions.

Dendritic cells (DCs)<sup>3</sup> are APCs that initiate primary immune responses by activating naive Th cells (17, 18). In addition to presenting

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Received for publication March 16, 2004. Accepted for publication November 16, 2004.

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<sup>1</sup> This work was supported by Grant-in-Aid for Creative Scientific Research 13GS0009 and Grant 12215067 from the Ministry of Education, Science, Technology, Sports, and Culture of Japan as well as by the Program for Research on Pharmaceutical and Medical Safety and the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research in Japan.

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand.

antigenic peptides to TCRs and activating naive Th cells, DCs provide an additional signal that induces Th cell polarization and thereby determines the nature of the immune response (19, 20). The *in vivo* transfer of DCs that have been pulsed *in vitro* with Ag efficiently primes Ag-specific Th cells (21) and induces strong Ab responses (22). Thus, *in vivo* DC transfer can be a useful, nonadjuvant-based method to induce Ag-specific immune responses.

Two distinct DC subsets in murine spleen have been characterized with regard to their expression of the CD8 $\alpha$  homodimer and the type of Th response they induce. *In vivo* studies have shown that the adoptive transfer of Ag-pulsed CD8 $\alpha^+$  DCs triggers the development of Th1 cells, whereas CD8 $\alpha^-$  DC transfer induces a Th2-type response to soluble protein Ags (23, 24). We also recently reported that each DC subset induces a distinct Ab profile that reflects its ability to prime specific Th responses (25).

In the present work we used the Ag-pulsed DC transfer system to determine whether Ag-specific Th1 cells can be primed in the presence of a Th2 response and whether this can reverse the overall Th phenotype of the immune system. Whether the pre-existence of an Ag-specific Th1 response prevents subsequent Th2 priming was also examined.

## Materials and Methods

### Animals

BALB/c mice were purchased from Seac Yoshitomi. DO11.10 mice on the BALB/c background, which are transgenic for a TCR recognizing chicken OVA peptide (OVA<sub>323-339</sub>) in the context of the MHC class II molecule I-A<sup>d</sup> (26), were a gift from Dr. S. Sakaguchi (Kyoto University, Kyoto, Japan). All mice were maintained in our pathogen-free facility and cared for in accordance with the institutional guidelines for animal welfare.

### Reagents

Murine rGM-CSF and rIL-2 were provided by Kirin Brewery and Shionogi Pharmaceutical, respectively. Murine rIFN- $\gamma$  and rIL-4 were purchased from R&D Systems. OVA and keyhole limpet hemocyanin (KLH) preparations that contain minimum levels of endotoxin were purchased from Seikagaku Kogyo and Calbiochem, respectively.

### Preparation of DCs

DCs were prepared as described, but with a minor modification (23). Briefly, spleens of 8- to 12-wk-old BALB/c mice were digested with collagenase D (Roche), filtered through a nylon sieve, and further dissociated in Ca<sup>2+</sup>-free HBSS containing 10 mM EDTA. The cells were resuspended in HistoDenz solution (Sigma-Aldrich) and separated into low and high density fractions by centrifugation at  $1700 \times g$  for 15 min. The low density cells were collected and incubated for 90 min in X-VIVO 15 (BioWhittaker) supplemented with 0.5% mouse plasma, 50  $\mu$ M 2-ME, and 20 ng/ml rGM-CSF. The nonadherent cells were washed off, and the remaining cells were cultured overnight in fresh medium containing 1 mg/ml OVA or KLH. The floating cells were collected, and the CD8 $\alpha^+$  DCs were positively selected using anti-CD8 MicroBeads and autoMACS (Miltenyi Biotec). From the unselected fraction, CD8 $\alpha^+$  DCs were further depleted, and the CD8 $\alpha^-$  DCs were positively enriched with anti-CD11c MicroBeads (Miltenyi Biotec). Flow cytometric analysis revealed that the purified fractions contained >96% CD8 $\alpha^+$ CD11c<sup>+</sup> and CD8 $\alpha^-$ CD11c<sup>+</sup> cells (data not shown).

### Preparation of naive T cells from DO11.10 transgenic mice

CD4<sup>+</sup> cells were positively selected from spleen cells of DO11.10 transgenic mice using Dynabeads mouse CD4 and DETACHaBEAD mouse CD4 (DynaL Biotec) according to the manufacturer's instructions. From the enriched CD4<sup>+</sup> cells, naive cells were positively selected with anti-CD62 ligand (CD62L) MicroBeads and autoMACS (Miltenyi Biotec).

### Immunization protocols

On day 0,  $2 \times 10^5$  OVA-pulsed DCs in 200  $\mu$ l of PBS were transferred *i.v.* into 8-wk-old BALB/c mice. A boost of 100  $\mu$ g of soluble OVA was given *i.v.* 7 days later, and the mice were killed on day 14. Sera were collected for the measurement of OVA-specific Ab titers, and spleens were removed for secondary stimulation. Alternatively, KLH-pulsed DCs were adminis-

tered, and the mice were killed on day 14 for secondary stimulation of splenocytes. To assess the influence of pre-existing Th1 or Th2 responses on the subsequent priming of the other Th subset, mice were injected on day -14 with  $2 \times 10^5$  Ag-pulsed DCs or PBS alone, and the other DC subset was given on day 0.

### Spleen cell stimulation

On day 14 spleens were removed, and single-cell suspensions were prepared. Cells ( $2 \times 10^6$ ) were cultured with or without the Ag (100  $\mu$ g/ml) in 48-well plates in X-VIVO 20 (BioWhittaker) supplemented with 0.5% mouse plasma and 50  $\mu$ M 2-ME. Supernatants were harvested 72 h later and stored at -40°C for subsequent cytokine analysis by ELISA.

### *In vivo* proliferation of naive Th cells induced by each DC subset

Naive DO11.10 Th cells ( $2 \times 10^6$ ) were transferred *i.v.* into BALB/c mice 1 day before the administration of OVA-pulsed DCs, and the mice were killed on day 14. The total number of splenocytes was counted, and the phenotype of the transferred DO11.10 Th cells was evaluated by labeling the cells with biotin-conjugated anti-clonotype Ab (KJ1-26; Caltag Laboratories), PE-conjugated anti-mouse CD62L (MEL-14; eBioscience), or FITC-conjugated anti-mouse CD44 (IM7; eBioscience), followed by incubation with allophycocyanin-streptavidin (BD Pharmingen). Anti-mouse CD16/CD32 mAb (2.4G2; BD Pharmingen) was used to block nonspecific binding to the FcRs before staining. The samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The numbers of naive and activated DO11.10 Th cells within a spleen were calculated by multiplying the total number of splenocytes by the percentage of KJ1-266-positive cells, then by the percentage of either CD62L<sup>high</sup>CD44<sup>low</sup> or CD62L<sup>low</sup>CD44<sup>high</sup> cells within the KJ1-26 positive fraction, respectively.

### *In vitro* priming of naive DO11.10 T cells with DC subsets

OVA-pulsed DCs ( $1 \times 10^4$  cells/well) and naive DO11.10 T cells ( $2 \times 10^5$  cells/well) were suspended in X-VIVO 15 supplemented with 0.5% mouse plasma, 50  $\mu$ M 2-ME, and 20 ng/ml rGM-CSF, then cultured in 96-well, U-bottom plates (Falcon; BD Biosciences). rIFN- $\gamma$  or rIL-4 was added in graded concentrations. On days 3, 4, and 6, the cultures were split and expanded in the presence of rIL-2 (35 U/ml). The cells were harvested on day 9, washed extensively, and counted, and the viable cells were tested for cytokine production. T cells ( $2 \times 10^5$  cells/well) were restimulated in a 96-well, U-bottom plate with 30-Gy irradiated BALB/c splenocytes ( $2 \times 10^5$  cells/well) with or without OVA (100  $\mu$ g/ml). Forty-eight hours after restimulation, supernatants were collected and stored at -40°C for subsequent analysis.

### Determination of Ab titers and cytokine levels by ELISA

Serum levels of OVA-specific Abs were determined as previously described (27). To measure OVA-specific IgE, serum IgE was absorbed to 96-well EIA/RIA plates (Corning Glass) coated with 2  $\mu$ g/ml anti-mouse IgE (R35-72; BD Pharmingen), and the bound Ab was detected by biotinylated OVA, followed by streptavidin-HRP (BD Pharmingen). To detect OVA-specific IgG1 and IgG2a, the sera were incubated in 96-well plates coated with OVA (50  $\mu$ g/ml), and the bound Abs were detected by biotin-conjugated anti-mouse IgG1 (A85-1; BD Pharmingen) and IgG2a (R19-15; BD Pharmingen), followed by streptavidin-HRP. The Ab titers were calculated by comparison with internal standards run in each assay. The anti-OVA IgE and IgG2a serum standards were obtained by pooling sera from mice immunized *i.p.* with OVA and Imject Alum (Pierce). Anti-OVA IgG1 mAb (OVA-14) purchased from Sigma-Aldrich was used as the IgG1 standard. Quantitative ELISAs for IFN- $\gamma$  and IL-4 in culture supernatants were conducted using OptEIA mouse cytokine sets (BD Pharmingen).

### Statistical analysis

Differences between two groups were examined for statistical significance using Student's *t* test for cytokine concentration and the Mann-Whitney *U* test for Ab titers. A value of *p* < 0.05 was considered significant.

## Results

### Induction of distinct Th and Ab responses by CD8 $\alpha^+$ and CD8 $\alpha^-$ DCs

We first evaluated the cytokine and Ab production profiles induced by the transfer of CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs. Thus, OVA-pulsed splenic CD8 $\alpha^+$  or CD8 $\alpha^-$  DCs were adoptively transferred into

syngeneic BALB/c mice on day 0, and a boost of soluble OVA was given i.v. 7 days later. Mice were killed on day 14, and OVA-specific Ab titers in serum and the cytokine production profile of splenocytes were determined. The transfer of CD8 $\alpha^+$  DCs resulted in a much higher ratio of IFN- $\gamma$ :IL-4 production by the splenocytes, whereas CD8 $\alpha^-$  DC transfer induced Th2 responses with a low IFN- $\gamma$ :IL-4 ratio (Fig. 1A). Moreover, OVA-specific IgG2a was only detected in the mice receiving CD8 $\alpha^+$  DCs, whereas OVA-specific IgE was only detected in mice given CD8 $\alpha^-$  DCs (Fig. 1B). OVA-specific IgG1 was detected in mice that received either DC subset, although slightly higher levels were detected in mice that were given CD8 $\alpha^-$  DCs. These Ab responses were Ag specific, because the injection of KLH-pulsed DCs, followed by a boost of soluble OVA, failed to induce the production of OVA-specific Abs (data not shown). OVA-specific Abs were not detected when mice were injected with OVA-pulsed DCs but did not receive the boost of soluble OVA (data not shown). The induction

of distinct Th responses through the transfer of CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs was confirmed using KLH as the Ag (Fig. 1C).

#### CD8 $\alpha^+$ DCs fail to prime a Th1 response in an Ag-specific Th2 environment

We next asked whether Ag-specific Th1 cells could be primed in the presence of an Ag-specific Th2 response, thereby skewing the overall Th phenotype of the immune response. Thus, we first introduced OVA-pulsed CD8 $\alpha^-$  DCs, then 14 days later we administered OVA-pulsed CD8 $\alpha^+$  DCs. This was followed by a boost with soluble OVA 7 days later, and mice were killed 7 days after this. When splenocytes from these mice were incubated with OVA, they failed to produce IFN- $\gamma$  and, instead, secreted a substantial amount of IL-4 (Fig. 2A). With regard to the serological response, OVA-specific IgG2a were not present in the sera of these

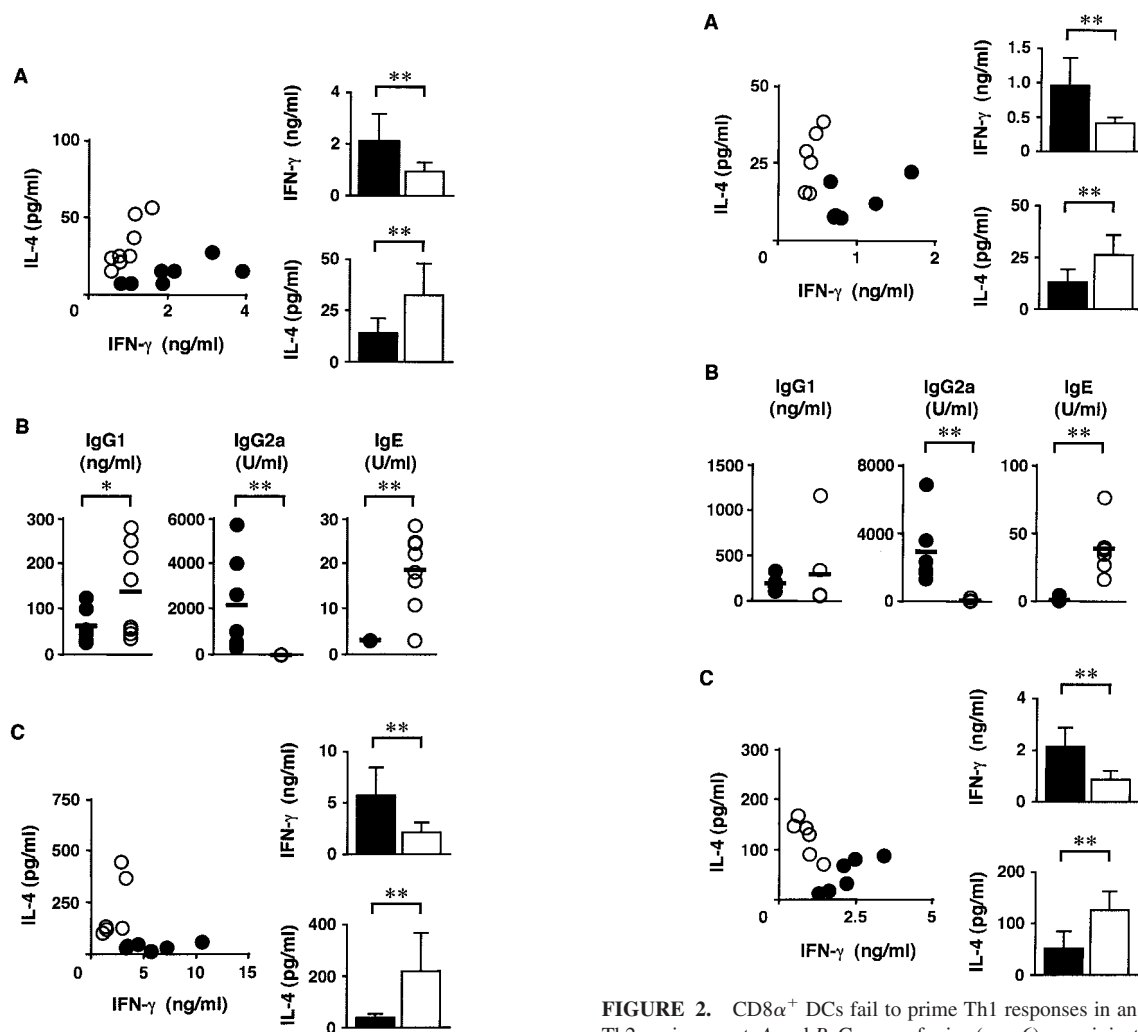


**FIGURE 1.** The transfer of Ag-pulsed CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs induces distinct types of Th and Ab responses. *A* and *B*, Groups of mice were given OVA-pulsed CD8 $\alpha^+$  ( $n = 7$ ; ●) or CD8 $\alpha^-$  ( $n = 8$ ; ○) DCs and a boost of soluble OVA 7 days later. The mice were killed on day 14, and their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (*A*) and the OVA-specific Ab titers (*B*). *C*, Groups of mice ( $n = 6$ ) were injected with KLH-pulsed CD8 $\alpha^+$  DCs (●) or CD8 $\alpha^-$  DCs (○), and the cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean  $\pm$  SD of each group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (by Student's *t* test for cytokine concentration and Mann-Whitney *U* test for Ab titers).

**FIGURE 2.** CD8 $\alpha^+$  DCs fail to prime Th1 responses in an Ag-specific Th2 environment. *A* and *B*, Groups of mice ( $n = 6$ ) were injected with  $2 \times 10^5$  OVA-pulsed CD8 $\alpha^-$  DCs (○) or PBS (●) on day -14, and  $2 \times 10^5$  OVA-pulsed CD8 $\alpha^+$  DCs were given on day 0. A boost of soluble OVA was given 7 days later, and the mice were killed on day 14. Their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (*A*) and the OVA-specific Ab titers (*B*). *C*, Groups of mice ( $n = 6$ ) were injected with  $2 \times 10^5$  KLH-pulsed CD8 $\alpha^-$  DCs (○) or PBS (●) on day -14, and  $2 \times 10^5$  KLH-pulsed CD8 $\alpha^+$  DCs were given on day 0. The cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean  $\pm$  SD of each group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (by Student's *t* test for cytokine concentration and Mann-Whitney *U* test for Ab titers).

mice; rather, high levels of OVA-specific IgE were detected (Fig. 2B). A similar phenomenon was observed when KLH was used as the Ag in place of OVA; namely, immunization with first KLH-pulsed CD8 $\alpha^+$  DCs and then KLH-pulsed CD8 $\alpha^+$  DCs resulted in splenocytes that secreted high levels of IL-4, rather than IFN- $\gamma$  (Fig. 2C).

#### CD8 $\alpha^-$ DCs can prime Th2 cells in the presence of an Ag-specific Th1 response

We then assessed whether a pre-existing, Ag-specific Th1 response would inhibit the subsequent development of Th2 cells specific for the same Ag. Thus, OVA-pulsed CD8 $\alpha^+$  DCs were administered before transfer of OVA-pulsed CD8 $\alpha^-$  DCs. This resulted in splenocytes that produced IL-4 along with IFN- $\gamma$  (Fig. 3A), namely, a mixed Th1/Th2 response. Moreover, both OVA-specific

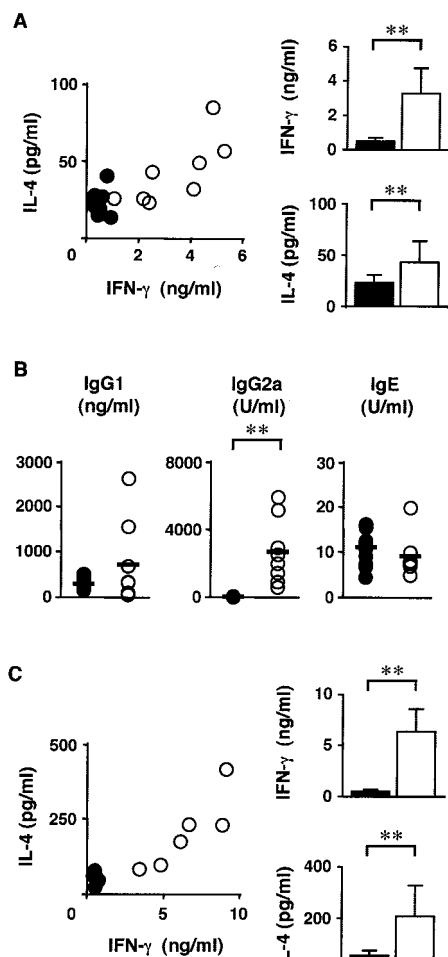


**FIGURE 3.** CD8 $\alpha^-$  DCs can prime Th2 cells in the presence of Ag-specific Th1 cells. **A** and **B**, Groups of mice were injected with  $2 \times 10^5$  OVA-pulsed CD8 $\alpha^+$  DCs ( $n = 8$ ;  $\circ$ ) or PBS ( $n = 11$ ;  $\bullet$ ) on day -14, and  $2 \times 10^5$  OVA-pulsed CD8 $\alpha^-$  DCs were given on day 0. A boost of soluble OVA was given 7 days later, and the mice were killed on day 14. Their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (**A**) and the OVA-specific Ab titers (**B**). **C**, Groups of mice ( $n = 6$ ) were injected with  $2 \times 10^5$  KLH-pulsed CD8 $\alpha^+$  DCs ( $\circ$ ) or PBS ( $\bullet$ ) on day -14, and  $2 \times 10^5$  KLH-pulsed CD8 $\alpha^-$  DCs were given on day 0. The cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean  $\pm$  SD of each group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (by Student's  $t$  test for cytokine concentration and Mann-Whitney  $U$  test for Ab titers).

IgG2a and IgE were generated by this immunization regimen (Fig. 3B). When KLH was used as the Ag, both IL-4 and IFN- $\gamma$  were again produced when the mice had been pretreated with CD8 $\alpha^+$  DCs (Fig. 3C).

#### CD8 $\alpha^+$ and CD8 $\alpha^-$ DCs prime similar numbers of naive Th cells

We speculated that the opposite effects of CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs preimmunization on subsequent Th priming by the other DC subset may reflect differences in their potential to prime absolute numbers of naive Th cells. In other words, should CD8 $\alpha^-$  DCs prime many more Ag-specific Th cells than CD8 $\alpha^+$  DCs, this might leave fewer Ag-specific naive Th cells after the transfer of CD8 $\alpha^-$  DCs, which might limit the subsequent Th1 priming induced by CD8 $\alpha^+$  DC transfer. To evaluate the potential of each DC subset to prime naive Th cells, naive DO11.10 Th cells were transferred into BALB/c mice 1 day before the injection of OVA-pulsed DC subsets, and the phenotype of the transferred Th cells in spleen was analyzed 14 days later. As shown in Fig. 4A, most DO11.10 Th cells remained CD62L<sup>high</sup> CD44<sup>low</sup> in the absence of DC transfer. However, after the transfer of either type of OVA-pulsed DC, about half the DO11.10 Th cells were activated and had acquired the CD62L<sup>low</sup> CD44<sup>high</sup> phenotype (Fig. 4A). When the absolute numbers of naive and activated Th cells in spleens were calculated, it became clear that the transfer of either DC subset left similar numbers of DO11.10 Th cells unprimed, and that these cells constituted the majority of the transferred DO11.10 Th cells (Fig. 4B). However, the transfer of CD8 $\alpha^-$  DCs did generate more activated Th cells (Fig. 4C). Thus, CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs do not differ in their potential to prime naive Th cells, and similar numbers of Ag-specific naive Th cells remain after the transfer of either DC subset. However, CD8 $\alpha^-$  DC-primed Th2 cells do proliferate more vigorously than CD8 $\alpha^+$  DC-primed Th1 cells.

#### Exogenous IL-4 abolishes the CD8 $\alpha^+$ DC-induced in vitro development of Th1 cells, but IFN- $\gamma$ fails to suppress the development of Th2 cells induced by CD8 $\alpha^-$ DCs

It is generally accepted that IFN- $\gamma$  from Th1 cells inhibits the development of Th2 cells, whereas IL-4 secreted from Th2 cells

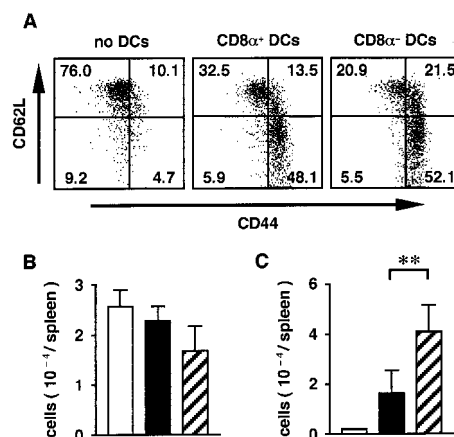


**FIGURE 4.** CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs prime similar numbers of naive Th cells. Naive DO11.10 Th cells were transferred i.v. into BALB/c mice 1 day before the administration of OVA-pulsed CD8 $\alpha^+$  DCs ( $n = 4$ ;  $\blacksquare$ ), CD8 $\alpha^-$  DCs ( $n = 6$ ;  $\boxplus$ ), or PBS ( $n = 3$ ;  $\square$ ). The mice were killed on day 14, and the phenotype of the KJ1-26<sup>+</sup> cells (**A**) and the absolute numbers of naive (**B**) or activated (**C**) KJ1-26<sup>+</sup> cells in spleens were determined. \*\*,  $p < 0.01$  (by Mann-Whitney  $U$  test).

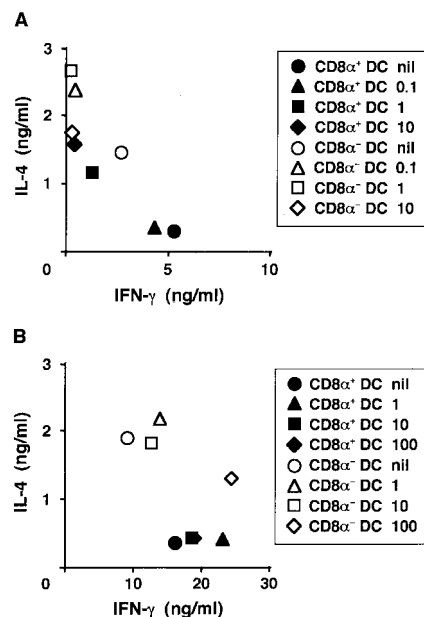


**FIGURE 5.** Effects of exogenous IL-4 and IFN- $\gamma$  on in vitro DC-induced Th cell differentiation. Naive DO11.10 T cells were primed with OVA-pulsed CD8 $\alpha^+$  (●, ▲, ■, and ◆) or CD8 $\alpha^-$  (○, △, □, and ◇) DCs. Graded doses of rIL-4 (A) or rIFN- $\gamma$  (B; nanograms per milliliter) were added at the same time. The primed T cells were restimulated 9 days later with 30-Gy irradiated BALB/c splenocytes and OVA, and the culture supernatants were obtained for assaying cytokine concentrations by ELISA. The data shown are representative of three independent experiments, all with similar results.

inhibits the priming of Th1 cells. However, our in vivo data indicate that whereas pre-existing Ag-specific Th2 cells do indeed effectively abolish the subsequent development of Th1 responses (Fig. 2), preprimed Ag-specific Th1 responses cannot suppress the subsequent priming of naive Th cells into the Th2 type (Fig. 3). We next evaluated the effect of adding exogenous IFN- $\gamma$  and IL-4 to cultures of naive DO11.10 cells during their in vitro priming by CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs. In the absence of these exogenous cytokines, CD8 $\alpha^+$  DCs induced the development of Th1 cells, whereas CD8 $\alpha^-$  DCs induced the development of Th2 cells (Fig. 5). The addition of 1 ng/ml rIL-4 to the priming culture abolished the priming of Th1 cells by CD8 $\alpha^+$  DCs and induced the development of Th2 cells (Fig. 5A). In contrast, as much as 100 ng/ml rIFN- $\gamma$  added to the priming culture only partially suppressed IL-4 production by CD8 $\alpha^-$  DC-primed Th cells, although the production of IFN- $\gamma$  was increased (Fig. 5B). These results suggest that the suppressive effects of IFN- $\gamma$  on the induction of Th2 cells by CD8 $\alpha^-$  DCs are limited, whereas IL-4 can overcome the Th1 priming signal provided by CD8 $\alpha^+$  DCs and effectively induce the development of Th2 cells.

## Discussion

Many current therapeutic approaches to allergic disorders focus exclusively on the amelioration of symptoms, and few primary measures to prevent the development or overcome the activity of allergen-specific Th2 cells have been proposed. However, advances in our understanding of the Th1/Th2 paradigm have raised the possibility that allergic disorders may be treated or prevented by inducing allergen-specific Th1 responses (10). To elucidate how Ag-specific Th1 and Th2 responses interact in vivo, we used a system of well-defined murine DC subset transfer to induce distinct Th responses. Two distinct DC subsets in murine spleen have

been characterized with regard to the expression of the CD8 $\alpha$  homodimer. There are about twice as many CD8 $\alpha^-$  DCs as CD8 $\alpha^+$  DCs (28, 29). The functional properties of each DC subset are not immutably fixed, and many additional factors contribute to their ability to regulate T cell priming (20). In particular, neither CD8 $\alpha^+$  nor CD8 $\alpha^-$  DCs can induce optimal T cell responses in their immature state, and their maturation is a prerequisite for both DCs to become potent activators of naive T cells. Moreover, although both DC subsets are in an immature state under steady state conditions, they become activated in response to pathogen-derived signals; overnight in vitro culture also activates these cells (30). Using ex vivo Ag-pulsed DCs for immunization eliminates the in vivo contribution of these factors and thus is not a physiological means to induce Ag-specific T cell responses; nevertheless, this method is useful because it obviates the need to administer artificial adjuvants and exogenous cytokines in vivo.

We found that the adoptive transfer of Ag-pulsed CD8 $\alpha^+$  DCs induced the development of Th1 responses and the production of IgG2a, whereas CD8 $\alpha^-$  DCs primed a Th2 response and induced the production of IgE (Fig. 1). Because IFN- $\gamma$  induces Ig class switching to IgG2a, and IL-4 enhances the production of IgE (31), our results indicate that in vivo transfer of each DC subset induces a distinct type of Th response, which then leads to a distinct Ab profile after a boost with soluble Ag. This is supported by the fact that OVA-specific Abs were not detected when mice were injected with OVA-pulsed DCs but did not receive a boost of soluble OVA (data not shown). Although the administration of soluble Ag is associated with the induction of tolerance in some circumstances, this is unlikely to have occurred in our experimental system, because boosting with soluble OVA resulted in increased levels of cytokine production by the in vitro splenocyte cultures (data not shown). Notably, substantial amounts of IgG1 Ab, which are generally considered to be associated with a Th2 response, were also produced after the transfer of CD8 $\alpha^+$  DCs, although a slightly higher level was induced by CD8 $\alpha^-$  DCs (Fig. 1B). With regard to this, one study has suggested that the production of IgG1 is not entirely Th2 dependent (32), and both Th1 and Th2 cells have been observed to induce similar levels of IgG1 (33). There was no significant correlation between levels of particular Ab isotypes in the sera and levels of the various cytokines that were produced by the in vitro splenocyte culture, which suggests that the way the Th cytokines function is complex. The ability of each DC subset to induce distinct Th responses was also confirmed in vitro (Fig. 5). Because this culture system was comprised of DCs and Th cells alone, this observation indicates that the two DC subsets issue distinct signals that lead to the differentiation of a particular Th cell phenotype. These observations together indicate that CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs induce distinct types of Th responses both in vivo and in vitro that effectively support the production of specific Ab isotypes in vivo.

We found that in the presence of an Ag-specific Th2 environment due to the transfer of Ag-pulsed CD8 $\alpha^-$  DCs, CD8 $\alpha^+$  DCs cannot induce Th1 responses (Fig. 2). However, the reverse is not true, because the Ag-specific Th1 response induced by the transfer of Ag-pulsed CD8 $\alpha^+$  DCs cannot effectively suppress subsequent priming of the Th2 response by CD8 $\alpha^-$  DCs. Rather, in this situation the production of IL-4 increased along with that of IFN- $\gamma$ , indicating a mixed Th1/Th2 response (Fig. 3). We showed that these discrepant abilities to block subsequent priming do not arise from the fact that CD8 $\alpha^-$  DCs prime so many T cells that too few are left for CD8 $\alpha^+$  DCs to prime a sufficient response, because CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs did not differ significantly in their potential to prime naive Th cells. In other words, similar numbers of Ag-specific naive Th cells remained after the transfer of each DC

subset (Fig. 4B). However, there were twice as many CD8 $\alpha$ <sup>-</sup> DC-primed Th2 cells compared with CD8 $\alpha$ <sup>+</sup> DC-primed Th1 cells (Fig. 4C), which means that CD8 $\alpha$ <sup>-</sup> DC-primed Th2 cells proliferate more vigorously than CD8 $\alpha$ <sup>+</sup> DC-primed Th1 cells. These findings are in accordance with the observation of an in vitro study that shows that CD8 $\alpha$ <sup>+</sup> DCs prime naive Th cells as well as CD8 $\alpha$ <sup>-</sup> DCs, but then induce the Th cells they activate to undergo Fas-mediated apoptosis (34). One conclusion of these latter observations may be that the failure of CD8 $\alpha$ <sup>+</sup> DCs to inhibit subsequent Th2 priming by CD8 $\alpha$ <sup>-</sup> DCs could be due in part to the inability of CD8 $\alpha$ <sup>+</sup> DCs to leave sufficient numbers of Th1 cells in vivo. However, we also found that exogenous IFN- $\gamma$  did not efficiently inhibit the potential of CD8 $\alpha$ <sup>-</sup> DCs to prime IL-4-producing cells (Fig. 5B), whereas the addition of IL-4 abolished the Th1 priming potential of CD8 $\alpha$ <sup>+</sup> DCs in vitro (Fig. 5A). Thus, our results indicate that there is a genuine discrepancy between Th1 and Th2 cells in their potential to inhibit the development of the other, because Th2 cells can effectively suppress Th1 development, but not vice versa.

It is generally accepted that IFN- $\gamma$  produced by Th1 cells and IL-4 produced by Th2 cells act antagonistically in Th cell differentiation (4, 35). IL-4 directly triggers the differentiation of Th2 cells (36, 37) and counteracts Th1 differentiation by down-regulating the expression of the IL-12R  $\beta$ <sub>2</sub>-chain on Th cells (38), hence blocking the IL-12 signal transduction pathway. In contrast, IFN- $\gamma$  suppresses IL-4-induced IL-4R gene expression by mRNA destabilization (39). However, although IL-4 can induce Th2 differentiation even in the presence of IL-12 (40, 41), IFN- $\gamma$  treatment of early developing Th2 cells does not lead to their reduced IL-4 or increased IFN- $\gamma$  production (38). Therefore, it is likely that exogenous IFN- $\gamma$  could not overcome the Th2-inducing signal provided by CD8 $\alpha$ <sup>-</sup> DCs in the absence of IL-12 (Fig. 5B), whereas IL-4 by itself was sufficient to counteract the Th1-inducing signal provided by CD8 $\alpha$ <sup>+</sup> DCs (Fig. 5A).

Our findings regarding the Th1/Th2 paradigm are important for our understanding of allergy pathogenesis and how we can prevent or treat allergies, because they suggest that allergen-specific Th1 responses cannot be easily primed once allergen-specific Th2 responses have evolved. Moreover, that CD8 $\alpha$ <sup>-</sup> DCs could induce the production of IL-4 and IgE after Th1 priming with Ag-pulsed CD8 $\alpha$ <sup>+</sup> DCs suggests that allergen-specific Th2 responses are likely to be primed even in the presence of allergen-specific Th1 cells, resulting in a mixture of Th1 and Th2 responses. These results are in contrast with studies reporting that a Th1 response inhibits Th2 cell development. Several possibilities may explain this discrepancy. First, we used in vitro Ag-pulsed DCs to induce a distinct type of Th response; these DCs may be different from those involved in infection and allergy. An infection may induce Th1 differentiation more effectively than the administration of CD8 $\alpha$ <sup>+</sup> DCs. There is the possibility that a single administration of CD8 $\alpha$ <sup>+</sup> DCs could not induce a sufficiently strong Th1 response and that larger numbers or repetitive administration of CD8 $\alpha$ <sup>+</sup> DCs may be required to prime enough Th1 cells to suppress the Th2 response induced by CD8 $\alpha$ <sup>-</sup> DCs. In addition, the administration of DCs at different time points may have altered the outcome. Although these possibilities cannot be excluded, we nevertheless believe that our observations reflect a true facet of the Th1/Th2 paradigm, because CD8 $\alpha$ <sup>+</sup> DCs induced a substantial amount of IFN- $\gamma$  production in splenocyte cultures, which proves the efficacy of Th1 induction to a certain extent. The inhibition of Th1 priming by preimmunization with CD8 $\alpha$ <sup>-</sup> DCs could be attributed to some type of tolerance induction. Indeed, high levels of IL-10 were produced by the ex vivo splenocyte cultures after the transfer of CD8 $\alpha$ <sup>-</sup> DCs and by Th cells primed with CD8 $\alpha$ <sup>-</sup> DCs in vitro

(data not shown). Thus, IL-10 produced by Th2 cells after an encounter with CD8 $\alpha$ <sup>+</sup> DCs may have inhibited Th1 priming.

It is now believed that the original Th1/Th2 concept regarding allergy pathogenesis was oversimplified. Recent studies report that Th1 as well as Th2 cytokines are up-regulated in atopic patients (42, 43). Although administration of allergen-specific Th1 cells inhibited Th2-cell-mediated asthma in some animal models (44, 45), other studies reported cooperation between allergen-specific Th1 and Th2 cells in the pathogenesis of allergic disorders (46, 47). Moreover, a recent study reported that both Th1 and Th2 type allergen-specific responses were enhanced in atopic children, along with a decreased Th1 response to nonspecific mitogens (48). This suggests the importance of universal Th1 skewing, rather than introducing an allergen-specific Th1 response to prevent allergic disorders. Another report has shown that although the cytokine profiles of allergen-specific Th cells from nonallergic subjects and from subjects who have outgrown the allergy are indeed Th1-skewed, the absolute numbers of these cells are extremely low compared with those in allergic patients (49). Therefore, it seems that the allergen-specific Th1 responses in nonatopic individuals are associated with nonresponsiveness to allergens. Moreover, several studies indicate an association between a Th1-inducing stimulus and the induction of tolerance. For example, intestinal bacterial flora are needed for the induction of oral tolerance (50, 51), and treatment with *Mycobacterium vaccae* generates IL-10-producing regulatory T cells (52). It was also shown that regulatory T cells selectively express TLRs and are activated by LPS (53). These observations indicate that the antiallergic effects of Th1-inducing agents cannot be attributed solely to the priming of allergen-specific Th1 cells; rather, they may involve the induction of tolerance.

In conclusion, when we used a transfer system of well-defined murine DC subsets to induce specific types of Th cells, we found a discrepancy between Th1 and Th2 cells in their potential to inhibit the development of the other. Th1 priming was abolished in the presence of Ag-specific Th2 cells, but Th1 cells could not inhibit subsequent priming of Th2 cells. Our results suggest that the induction of allergen-specific Th1 cells, even before allergy sensitization, will not be able to prevent the development of atopic disorders. Additional research into the complex mechanisms governing the initiation of and protection from allergic disorders is needed before other therapeutic approaches aimed at blocking the development or activity of allergen-specific immune cells can be devised. In particular, it will be important to determine how healthy individuals respond to allergens, because this will, in conjunction with our rapidly expanding understanding of allergy pathogenesis, provide new insights into the immune mechanisms that mediate resistance to allergies.

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