to a Th1-inducing pathogen up-regulates their expression of CD40 and enhances their potential to produce IL-12 in response to CD40 engagement (28–30). This reveals an attractive mechanism by which the development of a Th1 response can be augmented. Consistent with these observations, several studies revealed the importance of CD40-CD154 interactions in the development of anti-infection Th1 responses (31–34). However, recent studies have revealed that the CD40-CD154 interaction is also critically involved in the development of Th2 responses (35–38). Thus, the role that this costimulatory interaction plays in the induction of an appropriate immune response is complex.

To date, there are several reports concerning the role of CD40-CD154 interactions in the CTL-priming function of the $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets (16, 17). However, little is known about how each DC subset participates in naive Th cell priming. In particular, the role they play in regulating Th cell differentiation is unclear. In this study, we report on the new experimental systems that we developed to evaluate Th cell priming by $CD8\alpha^+$ and $CD8\alpha^-$ DCs and the role the CD40-CD154 interaction plays in these activities.

We found that the adoptive transfer of each DC subset induces the production of a distinct Ab that reflects their potential to provoke a specific type of Th response in vivo. We also found that each DC subset could induce a specific type of Th response in vitro. The interruption of the CD40-CD154 interaction both in vivo and in vitro inhibited the induction of the Th1 response by $CD8\alpha^+$ DCs but had little effect on the Th2 response induced by $CD8\alpha^-$ DCs. Our results indicate that CD40-CD154 interactions play an important role in the development of $CD8\alpha^+$ DC-driven Th1 responses but not in $CD8\alpha^-$ DC-driven Th2 responses.

Materials and Methods

Mice

BALB/c and C57BL/6 6- to 12-wk-old mice were purchased from Seac Yoshitomi (Fukuoka, Japan). DO11.10 mice on the BALB/c background, transgenic for a TCR recognizing a chicken OVA peptide ($OVA_{323-339}$) in the context of the MHC class II molecule $I\text{-}A^d$, were a generous gift from Dr. S. Sakaguchi (Kyoto University, Kyoto, Japan). All mice were maintained in our pathogen-free facility and were cared for in accordance with the institutional guidelines for animal welfare.

Reagents and Abs

Murine rGM-CSF and rIL-2 were kindly provided by Kirin Brewery (Tokyo, Japan) and Shionogi Pharmaceutical (Osaka, Japan), respectively. Murine rIL-4 and rIL-12 were obtained from R&D Systems (Eugene, OR). OVA and keyhole limpet hemocyanin (KLH) preparations that contain minimum levels of endotoxin were purchased from Seikagaku Kogyo (Tokyo, Japan) and Calbiochem (La Jolla, CA), respectively. ChromPure human IgG (hu-IgG) and hamster IgG (ham-IgG) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Functional grade purified anti-CD40 (HM40-3) and anti-CD154 (MR1) mAbs were purchased from BD PharMingen (San Diego, CA) and eBioscience (San Diego, CA), respectively.

CD40 and CTLA4 fusion proteins

Recombinant adenovirus vectors containing CD40-Ig or CTLA4-Ig gene, which encode an extracellular portion of murine CD40 or CTLA4 linked to the Fc portion of human IgG, were prepared as described (39, 40). COS7 cells were transfected with these vectors and the chimeric proteins were purified from the culture supernatant.

Preparation of DCs

DCs were prepared as described but with a minor modification (13). Briefly, spleens of 8- to 12-wk-old BALB/c mice were digested with collagenase D (Roche Molecular Biochemicals, Mannheim, Germany), filtered through a nylon sieve and further dissociated in Ca^{2+}-free HBSS containing 10 mM EDTA. The cells were resuspended in HistoDenz solution (Sigma-Aldrich, St. Louis, MO) 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, Walkersville, MD) supplemented with 0.5% mouse plasma, 50 μM 2-ME, and 20 ng/ml rGM-CSF. Nonadherent cells were washed off and the remaining cells were cultured overnight in fresh medium containing 1 mg/ml OVA or KLH to allow the DCs to detach from the plastic dishes. Floating cells were collected and $CD8\alpha^+$ DCs were positively selected using anti-CD8 MicroBeads and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). From the negative fraction, $CD8\alpha^+$ DCs were further depleted and $CD8\alpha^-$ DCs were positively enriched with anti-CD11c MicroBeads (Miltenyi Biotec). Flow cytometric analysis revealed that each purified fraction contained >96% $CD8\alpha^+CD11c^+$ and $CD8\alpha^-CD11c^+$ cells (data not shown).

Preparation of naive T cells from DO11.10 transgenic mice

$CD4^+$ cells were positively selected from spleen cells of DO11.10 transgenic mice using Dynabeads mouse CD4 and DETACHaBEAD mouse CD4 (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. From the enriched $CD4^+$ cells, naive cells were positively selected using anti-CD62 ligand MicroBeads and autoMACS (Miltenyi Biotec).

Immunization protocols

For the cytokine production experiment, 2×10^5 KLH-pulsed DCs were transferred i.v. into 6- to 7-wk-old BALB/c mice on day 0. Spleens were removed for the cytokine production assay on day 14. For the Ab-production experiment, OVA-pulsed DCs were administered and a boost of 100 μg of soluble OVA was given i.v. on day 14. Mice were bled 7 days later to measure serum Ab titers. To interrupt the CD40-CD154 or CD28-CD80/CD86 interactions, 200 μg of CD40-Ig or CTLA4-Ig was administered i.v. on days 0, 2, and 4 after DC transfer. Control mice received an equivalent amount of hu-IgG.

Splenic $CD4^+$ T cell stimulation

Fourteen days after the transfer of KLH-pulsed DCs, spleens were removed and single-cell suspensions were prepared. A total of 3×10^7 cells from each mouse were pooled within each experimental group and $CD4^+$ cells were positively selected using anti-CD4 MicroBeads and autoMACS (Miltenyi Biotec). Purified $CD4^+$ cells (10^6 cells/well) and 30 Gy-irradiated BALB/c splenocytes (10^6 cells/well) were cocultured in 48-well plates in X-VIVO 20 (BioWhittaker) supplemented with 0.5% mouse plasma and 50 μM 2-ME with or without KLH (100 μg/ml). Supernatants were harvested 72 h later and stored at −40°C for subsequent cytokine analysis using ELISA.

T cell proliferation assay

OVA-pulsed DCs (2.5×10^3 cells/well) and naive DO11.10 T cells (5×10^4 cells/well) were suspended in X-VIVO 15 supplemented with 0.5% mouse plasma, 50 μM 2-ME, and 20 ng/ml rGM-CSF and then cocultured for 72 h in 96-well U-bottom plates (BD Falcon, Franklin Lakes, NJ). CTLA4-Ig, CD40-Ig, anti-CD154 Ab (MR1), hu-IgG, or ham-IgG was added at graded concentrations. For the allogeneic MLR, splenic $CD4^+$ cells (1×10^5 cells/well) from C57BL/6 mice were stimulated with graded numbers of BALB/c DCs. [*methyl*-^{3}H]Thymidine (0.5 μCi/well; Moravek Biochemicals, Brea, CA) was pulsed for the last 8 h, and cell proliferation was determined by measuring radioactivity incorporated into the DNA using MeltiLex solid scintillation counting on a MicroBeta TRILUX beta-emitter detection system (PerkinElmer Wallac, Boston, MA).

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

OVA-pulsed DCs (1×10^4 cells/well) and naive DO11.10 T cells (2×10^5 cells/well) were cocultured in 96-well U-bottom plates as for the T cell proliferation assay. Anti-CD154 Ab (MR1) or ham-IgG at 10 μg/ml was added. On days 3, 4, and 6, the cultures were split and expanded in the presence of rIL-2 (35 U/ml). Cells were harvested on day 9, washed extensively, counted, and viable cells were tested for cytokine production. T cells (2×10^5 cells/well) were restimulated in a 96-well U-bottom plate with 30 Gy-irradiated BALB/c splenocytes (2×10^5 cells/well) and OVA (100 μg/ml). After 48 h of this restimulation, supernatants were collected and stored at −40°C for subsequent analysis. Anti-CD154 Ab (MR1) was used instead of CD40-Ig because the addition of hu-IgG altered the cytokine production profile and could not be used as a control (data not shown).

Flow cytometric analysis

To determine the expression of surface molecules, sorted DCs were labeled with the following mAbs: PE-conjugated anti-CD4 (GK1.5; BD PharMingen), anti-CD40 (3/23; Caltag Laboratories, Burlingame, CA), anti-CD54

(3E2; BD PharMingen), anti-CD80 (16-10A1; BD PharMingen), anti-CD86 (GL1; BD PharMingen), anti-MHC class I (SF1-1.1; BD PharMingen), FITC-conjugated anti-MHC class II (M5/114.15.2; eBioscience), and isotype-matched controls (BD PharMingen). The Ag-uptake potential of DC subsets was evaluated by pulsing DCs with FITC-conjugated OVA (Molecular Probes, Minneapolis, MN). To analyze the expression kinetics of costimulatory molecules after DC-T interaction, cultured cells were dissociated in Ca^{2+}-free HBSS containing 10 mM EDTA and labeled with PE-conjugated anti-CD28 (37.51; *e*Bioscience), anti-CD40, anti-CD80, anti-CD86, anti-CD152 (UC10-4B9; *e*Bioscience) or anti-CD154 (MR1; *e*Bioscience) mAbs. Allophycocyanin-conjugated anti-CD4 mAb or biotin-conjugated anti-CD11c (HL3; BD PharMingen) mAb, followed by allophycocyanin-streptavidin (BD PharMingen), was used to distinguish DCs from T cells. Anti-mouse CD16/CD32 mAb (2.4G2; BD PharMingen) was used to block nonspecific binding to the Fc receptor before staining. Samples were analyzed using a FACSCaliber flow cytometer and CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Stimulation of DC subsets for cytokine production

Sorted DC subsets were suspended in X-VIVO 15 supplemented with 0.5% mouse plasma, 50 μM 2-ME, 20 ng/ml rGM-CSF, and 10 ng/ml rIL-4 and then stimulated in 96-well U-bottom plates (2×10^5 cells/well) with 20 μg/ml anti-CD40 mAb (HM40-3) or 100 ng/ml LPS (Sigma-Aldrich). Supernatants were collected 24 h later and stored at $-40°C$ for subsequent analysis.

ELISA determination of Ab titers and cytokine levels

Serum levels of OVA-specific Abs were determined as previously described but with minor modifications (41). To measure OVA-specific IgE, serum IgE was absorbed to 96-well EIA/RIA plates (Corning, Corning, NY) coated with 2 μg/ml anti-mouse IgE mAb (R35-72; BD PharMingen) and the bound Abs were detected by biotinylated OVA, followed by streptavidin-HRP conjugate (BD PharMingen). To detect OVA-specific IgG1 and IgG2a, sera were incubated in 96-well plates coated with OVA (50 μg/ml) and bound Abs were detected using biotin-conjugated anti-mouse IgG1 (A85-1; BD PharMingen) and IgG2a (R19-15; BD PharMingen) mAbs, followed by streptavidin-HRP conjugate. The Ab titers were calculated by comparison with internal standards run in each assay. Anti-OVA IgE and IgG2a serum standards were obtained by pooling sera from mice immunized i.p. with OVA and Imject Alum (Pierce, Rockford, IL). Anti-OVA IgG1 mAb (OVA-14; Sigma-Aldrich) was used as a standard. Quantitative ELISAs for IFN-γ, IL-4, and IL-10 in culture supernatants were conducted using OptEIA mouse cytokine sets (BD PharMingen). IL-12p70 in culture supernatants was quantified using the DuoSet ELISA Development kit (R&D Systems).

Statistical analysis

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

Results

The CD40-CD154 interaction is only involved in the induction of Th1 responses by CD8α^+ DCs in vivo

We first assessed the role that the CD40-CD154 interaction plays during DC-induced splenic Th cell polarization in vivo. KLH-pulsed CD8α^+ and CD8α^- DCs were transferred into naive mice and 14 days later their splenic CD4$^+$ cells were cultured in the presence of KLH. The culture supernatants were then assessed for cytokine production. The KLH-pulsed CD8α^+ DCs induced a much higher level of IFN-γ production, whereas CD8α^- DCs preferentially induced the production of IL-4 and IL-10 (Fig. 1*A*). To interrupt the CD40-CD154 or CD28-CD80/CD86 costimulatory interactions, CD40-Ig or CTLA4-Ig was injected three times at 2-day intervals starting on the day of the DC transfer. Administration of CTLA4-Ig inhibited the IFN-γ and IL-4/IL-10 production by the splenic CD4$^+$ cells of the mice that had received the CD8α^+ and CD8α^- DCs, respectively (Fig. 1*B*). Administration of CD40-Ig also inhibited the IFN-γ production of the splenic CD4$^+$ cells induced by CD8α^+ DC-transfer (Fig. 1*C*). In contrast, the IL-4 and IL-10 production by CD8α^- DC-transferred mice

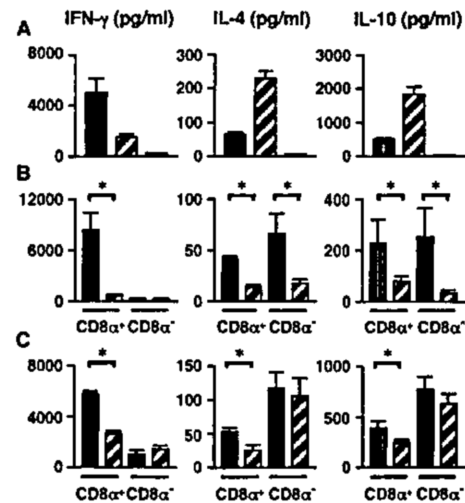

FIGURE 1. Effect of CTLA4-Ig and CD40-Ig on CD8α^+ DC-induced and CD8α^- DC-induced Th responses. *A*, Groups of BALB/c mice ($n = 3$) were injected with KLH-pulsed CD8α^+ DCs (■), CD8α^- DCs (▨), or PBS (□). Fourteen days later, CD4$^+$ cells were positively selected from the pooled splenocytes of each experimental group and stimulated with irradiated BALB/c splenocytes and KLH. Culture supernatants were harvested 72 h later and assayed for cytokine concentrations by ELISA. *B* and *C*, Groups of mice ($n = 3$) were immunized with KLH-pulsed CD8α^+ DCs or CD8α^- DCs and injected with CTLA4-Ig (*B*) or CD40-Ig (*C*) (▨) three times at 2-day intervals. Control mice received hu-IgG (■). The cytokine production of the splenic CD4$^+$ cells was determined as in *A*. The data shown are the mean ± SD of triplicate wells and are representative of three independent experiments with similar results. *, $p < 0.05$ by Student's *t* test.

remained unchanged (Fig. 1*C*). These results indicate that while CD80/CD86-CD28 interactions are required for the Th cell priming by both CD8α^+ and CD8α^- DCs, CD40-CD154 interactions are only required for the induction of the Th1 response by CD8α^+ DCs in vivo.

It has been reported that the in vivo transfer of Ag-pulsed DCs efficiently induces an Ag-specific Ab response (42). We modified this system and evaluated the effect of costimulation blockade on the Ab production profile induced by CD8α^+ and CD8α^- DCs. OVA-pulsed DCs were adoptively transferred into syngeneic BALB/c mice and a boost of soluble OVA was given i.v. 14 days later. The mice were bled 7 days later and the OVA-specific Abs in the sera were measured by ELISA. As shown in Fig. 2*A*, OVA-specific IgG2a was detected specifically in the mice that had received the CD8α^+ DCs whereas OVA-specific IgE was detected in mice given CD8α^- DCs. Similar levels of OVA-specific IgG1 were induced by either DC subset. When the dose of OVA that was used to Ag-pulse the DC was titrated from 30 μg/ml to 1 mg/ml, dose-dependent alterations in the levels of Ab production but no changes in the isotype profile were observed (data not shown). The Ab response was Ag-specific because injection of KLH-pulsed DCs, followed by a boost of soluble OVA, failed to induce the production of OVA-specific Abs (data not shown). In addition, OVA-specific Abs were not detected when mice were given an injection of OVA-pulsed DCs but no boost of soluble OVA (Fig. 2*A*). Because IFN-γ induces Ig-class switching to IgG2a, whereas IL-4 enhances the production of IgE, our results indicate that each DC subset induces a distinct Ab response that reflects their potential to prime a distinct type of Th response.

Consistent with the cytokine production inhibition patterns, all Ab classes induced by either DC subset were inhibited by

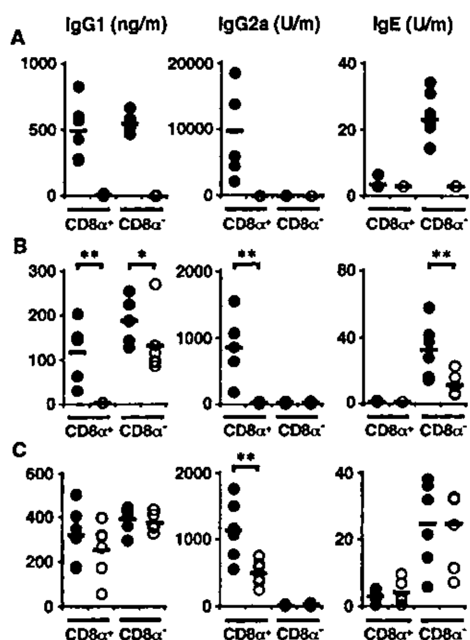

FIGURE 2. Effect of CTLA4-Ig and CD40-Ig on CD8α^+ DC-induced and CD8α^- DC-induced Ab responses. *A*, Groups of mice (n = 6–7 per group) received OVA-pulsed CD8α^+ or CD8α^- DCs and a boost of soluble OVA (●) or PBS (○) 14 days later. Sera were collected 7 days later and assayed for OVA-specific Ab isotype titers by ELISA. *B* and *C*, Groups of mice (n = 5–6 per group) received OVA-pulsed CD8α^+ or CD8α^- DCs and were injected with CTLA4-Ig (*B*) or CD40-Ig (*C*) (○) three times at 2-day intervals. Control mice received hu-IgG (●). A boost of soluble OVA was given 14 days after the DC transfer and serum levels of OVA-specific Ab isotype titers were assayed as in *A*. Each circle represents the titer of a single mouse, and bars show mean values for each group. *, $p < 0.05$; **, $p < 0.01$ by Mann-Whitney *U* test.

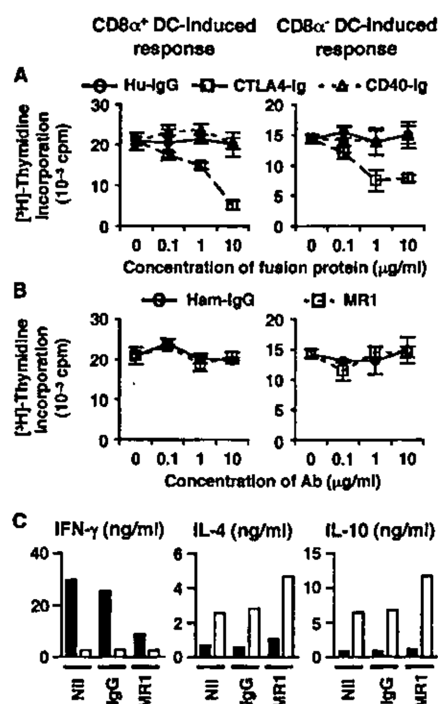

FIGURE 3. The CD40-CD154 interaction is not required for the proliferation of naive Th cells, but is indispensable for the induction of Th1 cells by CD8α^+ DCs in vitro. *A* and *B*, Naive DO11.10 CD4$^+$ T cells were cultured for 72 h with OVA-pulsed CD8α^+ (*left*) or CD8α^- (*right*) DCs. CTLA4-Ig, CD40-Ig (*A*) or anti-CD154 Ab (MR1) (*B*) was added to the cultures at graded concentrations. Proliferation was measured by pulsing the cultures with [^{3}H]thymidine for the last 8 h. *C*, Naive DO11.10 splenic CD4$^+$ T cells were primed with OVA-pulsed CD8α^+ (■) or CD8α^- (□) DCs in the presence or absence of the anti-CD154 Ab (MR1) or ham-IgG. The primed T cells were restimulated with irradiated splenocytes and OVA. Culture supernatants were harvested 48 h later and assayed for cytokine concentrations using ELISA. The data shown are representative of four (*A* and *B*) or six (*C*) independent experiments, all with similar results.

CTLA4-Ig (Fig. 2*B*) but CD40-Ig only inhibited the production of IgG2a induced by the CD8α^+ DCs (Fig. 2*C*). CD40-Ig did not affect the levels of IgG1 induced by the CD8α^+ DCs or the IgG1 and IgE Abs induced by CD8α^- DCs (Fig. 2*C*). These results further support the view that CD40-CD154 interactions are only required for the induction of the Th1 response by CD8α^+ DCs in vivo.

CD40-CD154 interactions are not required for DC-induced proliferation of Th cells but are indispensable for effective Th1 priming by CD8α^+ DCs in vitro

Naive Th cells must receive a defined series of activation and differentiation signals before they can mount appropriate immune responses. Thus, Th cell priming could be inhibited at two distinct levels, namely, at their activation or at their differentiation. The inhibition of their activation is likely to reduce the proliferation of Th cells but inhibition of their differentiation may not interfere with their proliferation. To elucidate the roles the CD40-CD154 interaction plays in the DC-primed proliferation and differentiation of Th cells, we used DO11.10 T cells in an in vitro assay system.

Thus, naive Th cells from DO11.10 mice were stimulated with OVA-pulsed DCs and their proliferative response was determined using the thymidine incorporation assay. As expected, T cells proliferated abundantly in response to both DC subtypes (Fig. 3, *A* and *B*). This response was Ag-specific because DCs not pulsed with OVA failed to induce any significant proliferation of DO11.10 T cells (data not shown). Although CTLA4-Ig dose-dependently inhibited the proliferation of Th cells induced by both the CD8α^+ (Fig. 3*A*, *left*) and CD8α^- DCs (Fig. 3*A*, *right*), CD40-Ig did not suppress the proliferation induced by either subset (Fig. 3*A*). The anti-CD154 mAb MR1, another agent commonly used to block the CD40-CD154 interaction, also failed to inhibit the proliferative response (Fig. 3*B*).

We then investigated whether interfering with the CD40-CD154 interaction would reduce the Th differentiation induced by the DC subsets. Thus, we determined the cytokine production profile of DO11.10 CD4$^+$ T cells primed by OVA-pulsed DCs in the presence or absence of the anti-CD154 mAb MR1. When restimulated with OVA and irradiated BALB/c splenocytes as APCs, CD8α^+ DC-primed T cells primarily produced IFN-γ whereas IL-4 and IL-10 were preferentially produced by CD8α^- DC-primed T cells (Fig. 3*C*). However, when the MR1 Ab was present during the priming, the production of IFN-γ by CD8α^+ DC-primed T cells was reduced (Fig. 3*C*). In contrast, the IL-4 and IL-10 production by CD8α^- DC-primed T cells was not affected by MR1 (Fig. 3*C*). Taken together, it appears that although the CD80/CD86-CD28 interaction is required for the DC-induced activation and proliferation of naive T cells, the CD40-CD154 interaction is only required for the CD8α^+ DC-induced differentiation of Th1 cells.

CD8α^+ and CD8α^- DCs express similar levels of costimulatory molecules

We next analyzed the Ag-uptake potential and the surface expression of costimulatory molecules on CD8α^+ and CD8α^- DCs.

Consistent with previous reports (43, 44), both DC subsets showed extensive uptake of OVA and expressed similar levels of CD40 as well as CD54, CD80, CD86, MHC class I and class II molecules after their initial isolation (Fig. 4*A*). About one-half of the CD8α^- DCs expressed CD4, whereas the CD8α^+ DCs did not (Fig. 4*A*). In addition, both DC subsets induced a vigorous allogeneic MLR (Fig. 4*B*), although CD8α^- DCs induced slightly higher response (9).

CD8α^+ and CD8α^- DCs express similar levels of CD40 after interaction with Th cells and induce similar levels of CD154 expression on the Th cells they activate

There remains a possibility that the differential requirement of CD40-CD154 interaction by the two DC subsets merely reflects the difference in the surface expression of these molecules after DC-T cell interaction, or that it may be due to an alteration in the expression of other costimulatory molecules, such as CD28-CD80/CD86. We tested this and found that after interacting with Th cells, the expression of CD40 on each DC subset was equally elevated (Fig. 5*A*). Moreover, similar levels of CD154 expression were induced on Th cells by either DC subset (Fig. 5*B*). The expression of CD80 and CD86 on DCs, as well as that of CD28 and CD152 on Th cells, was also not altered by interfering with the CD40-CD154 interaction during Th cell priming by the DCs (Fig. 5, *C–E*).

Selective production of cytokines by CD8α^+ DCs after CD40 triggering

It has been suggested that DC-derived IL-12 plays a crucial role in determining the Thl/Th2-promoting capacity of the CD8α^+ and CD8α^- DCs, and that IFN-γ promotes a Th1 response by inducing IL-12 production from CD8α^+ DCs whereas IL-10 promotes a Th2 response by inhibiting the IL-12 production by CD8α^- DCs (45). Consequently, we analyzed the cytokine production of CD8α^+ and CD8α^- DCs when they were cultured in the presence of an agonistic anti-CD40 mAb. CD8α^+ DCs produced IL-12 and IFN-γ after this CD40 triggering (Fig. 6). However, the CD8α^+ DCs also produced substantial amounts of IL-10 regardless of CD40 signaling (Fig. 6). In contrast, CD8α^- DCs did not produce either of these cytokines in response to CD40 triggering, and this is not because they produce IL-10 that suppresses their IL-12 production. Notably, neither DC subset produced IL-12 in response to LPS stimulation (Fig. 6). These results show that the two DC subtypes differ fundamentally in the cytokines they produce once their CD40 molecules have been ligated, and suggest that the distinct Th

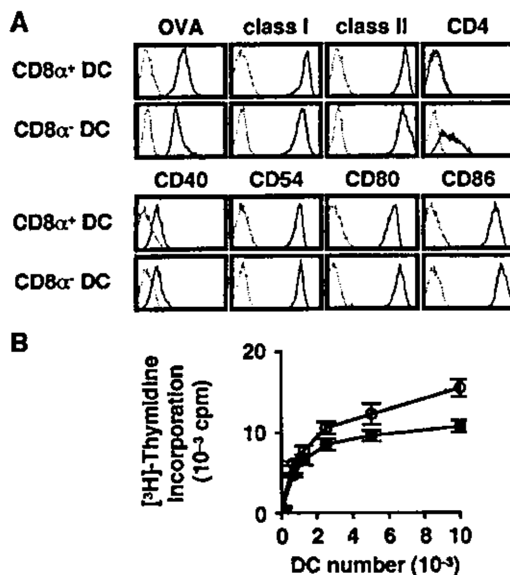

FIGURE 4. CD8α^+ and CD8α^- DCs express similar levels of costimulatory molecules. *A*, CD8α^+ and CD8α^- DCs were pulsed with FITC-OVA and analyzed for Ag-uptake potential. Their surface expression of CD4, CD40, CD54, CD80, CD86, MHC class I and class II molecules was also evaluated. The dotted lines represent DCs pulsed with unlabeled OVA or stained with isotype controls. *B*, Graded numbers of CD8α^+ (●) and CD8α^- DCs (O) were cultured with C57BL6 splenic CD4$^+$ cells for 72 h. Proliferation was measured by pulsing the cultures with [^{3}H]thymidine for the last 8 h. The data shown are representative of at least three independent experiments, all with similar results.

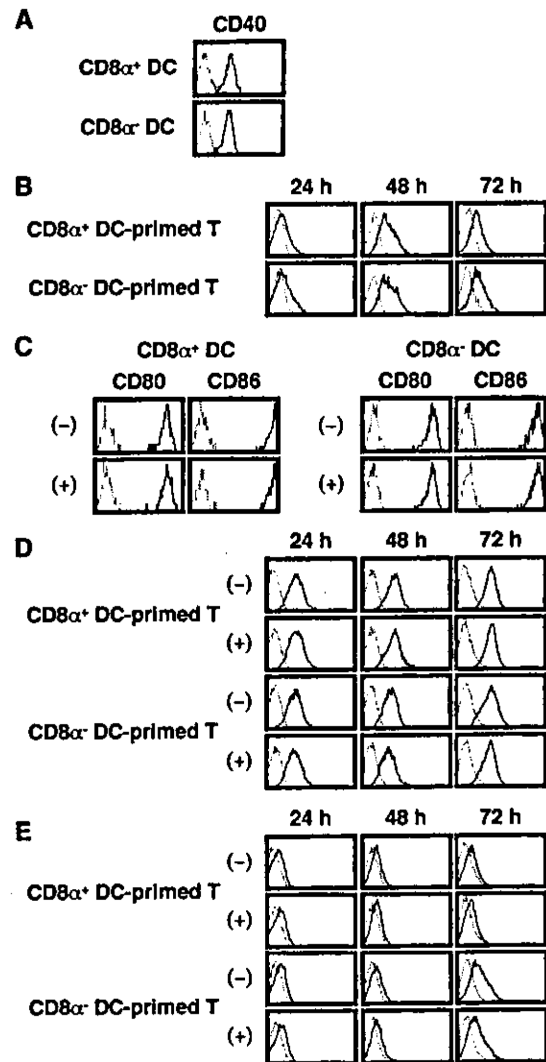

FIGURE 5. CD8α^+ and CD8α^- DCs express similar levels of CD40 after interaction with Th cells and induce similar levels of CD154 expression. *A*, OVA-pulsed CD8α^+ and CD8α^- DCs were cultured with naive DO11.10 CD4$^+$ T cells and surface expression of CD40 was analyzed 24 h later. *B*, Naive DO11.10 T cells were primed with OVA-pulsed CD8α^+ or CD8α^- DCs and surface expression of CD154 was analyzed at the indicated time points. *C*, OVA-pulsed DCs were cultured with naive DO11.10 T cells with (+) or without (−) anti-CD154 blocking mAb (MR1) and surface expression of CD80 and CD86 was analyzed 24 h later. *D* and *E*, Naive DO11.10 T cells were primed with OVA-pulsed DCs as in *C* and surface expression of CD28 (*D*) and CD152 (*E*) was analyzed at the indicated time points. The data shown are representative of at least three independent experiments, all with similar results.

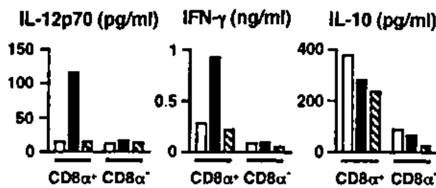

FIGURE 6. Selective production of cytokines by CD8α^+ DCs after CD40 triggering. Sorted DC subsets were left untreated (□) or stimulated either with an agonistic anti-CD40 mAb (■) or LPS (▨). The culture supernatants were harvested 24 h later and assayed for cytokine concentrations by ELISA. The data shown are representative of three independent experiments, all with similar results.

differentiation associated with each DC subtype is due to a basic difference in their response to CD40 ligation.

Discussion

Murine DCs isolated from the spleen fall into two distinct subsets with respect to their expression of the CD8 α-chain and the type of Th response they induce. Two in vivo studies have revealed that injection of CD8α^+ DCs triggers the development of Th1 cells, whereas injection of CD8α^- DCs induces a Th2-type response to soluble Ags (12, 13). With regard to the induction of humoral immunity, it was found that the selective expansion of either DC subset in vivo by the administration of growth factors results in increased Ab titers with skewed isotype profiles (12). However, the types of Ab responses that are induced by each DC subset remained to be determined. As Th1 and Th2 cells induce the production of distinct Ab isotypes (46), and the transfer of Ag-pulsed DCs efficiently induces an Ag-specific Ab response after a boost of soluble Ag in vivo (42), we speculated that the transfer of each DC subset would lead to the production of a distinct class of Abs that would reflect their potential to provoke a specific type of Th response. As shown in Fig. 2*A*, CD8α^+ and CD8α^- DCs specifically induced Ag-specific IgG2a and IgE production, respectively. This was in accord with the cytokine production profile of the splenic CD4$^+$ T cells obtained from mice transferred with each DC subset because CD8α^+ DCs induced much higher levels of IFN-γ, whereas IL-4 and IL-10 production was preferentially induced by CD8α^- DCs (Fig. 1*A*). Of note, similar levels of Ag-specific IgG1 Abs, which are often viewed as being associated with the Th2 response, were induced by both DC subsets (Fig. 2*A*). In this respect, one report has indicated that IgG1 production is not entirely Th2-dependent (47), and another has revealed that both Th1 and Th2 cells induce similar levels of IgG1 production (46). We also showed that each DC subset could induce a distinct type of Th response in vitro (Fig. 3*C*). Notably, the distinct effect of these subsets on Th cell differentiation, in particular the induction of Th2 cells by CD8α^- DCs, was confirmed only when U-bottom but not flat-bottom plates were used for the priming (data not shown). This suggests that close cell-to-cell contact between DCs and Th cells, or among the Th cells themselves is important for the effective differentiation of Th cells. As the culture system was comprised of DCs and Th cells, these in vitro observations also reveal that DCs actually provide a distinct signal that leads to the differentiation of Th cells. Taken together, our results indicate that CD8α^+ and CD8α^- DCs induce a distinct type of Th response both in vivo and in vitro that account for the different effects of these subsets on the production of specific Ab isotypes in vivo.

It is now well established that CD40-CD154 interactions play an important role in the priming of CD4$^+$ Th cell by DCs (18–20). In the present study, we found that the CD40-CD154 interaction is preferentially required for the induction of a Th1 response by CD8α^+ DCs but not for the induction of a Th2 response by CD8α^- DCs in vivo (Fig. 1*C*). These effects of CD40-Ig administration in vivo on the cytokine production profile were reflected in a selective reduction in the IgG2a Ab class that is produced by the transfer of CD8α^+ DCs (Fig. 2*C*). CD40-Ig had no effect on the Ab profile generated by CD8α^- DCs (Fig. 2*C*). In accordance with these in vivo results, CD40-CD154 blockade inhibited the Th1 differentiation of Ag-specific naive Th cells stimulated by CD8α^+ DCs in vitro (Fig. 3*C*). We also noted that the DC-induced proliferation of naive Th cells was not inhibited by interrupting the CD40-CD154 interaction (Fig. 3, *A* and *B*). Thus, it appears that the CD40-CD154 costimulatory pathway is required for the CD8α^+ DC-induced differentiation of Th1 cells but not for the activation of naive Th cells or the CD8α^- DC-induced differentiation of Th2 cells.

There was a possibility that the different requirement of CD40-CD154 interaction by the two DC subsets merely reflects the difference in the surface expression of these molecules. However, we found that CD8α^+ and CD8α^- DCs expressed similar levels of CD40 before (Fig. 4*A*) and 24 h after (Fig. 5*A*) their interaction with Th cells. Analysis at later time points in the in vitro culture was not performed because only a few cells were positive for CD11c by that stage. In addition, both the CD8α^+ and CD8α^- DC subsets induced similar levels of CD154 expression on naive Th cells (Fig. 5*B*) and the addition of exogenous IL-12 to the culture did not have a significant effect on their CD154 expression (data not shown). Notably, some studies have reported that activated Th cells under Th1-inducing conditions preferentially express CD154 (48, 49). However, these studies used different methods to stimulate the Th cells and it is thus difficult to determine why their observations differ from our own. We also found that expression of CD80/CD86 on DCs, as well as CD28/CD152 on Th cells, was not altered by blocking CD40-CD154 interaction during Th cell priming by the two DC subsets (Fig. 5, *C–E*).

The importance of CD40-CD154 interactions in Th cell priming was first revealed by studies on CD154 knockout mice, which are characterized by defective Th cell responses to protein Ags (50). The CD154-deficient Th cells do not undergo clonal expansion and enter the cell cycle, which suggests a probable defect at an early stage of T cell activation. Because constitutive CD80/CD86-CD28 costimulation appears to restore the potential of CD154-deficient mice to prime Th cells in some systems (51, 52), and DCs upregulate CD80/CD86 molecules upon CD40 ligation (21), lack of CD40-dependent DC activation is likely to be responsible for the defects in Th cell priming seen in CD154-deficient mice. As CD8α^+ and CD8α^- DCs already express high levels of CD80 and CD86 (Fig. 4*A*), these molecules may provide sufficient costimulation that allows the activation of naive Th cells and compensates for the lack of CD40-CD154 signaling in our experimental system. In support of this speculation, we found that blockade of CD80/CD86-CD28 interaction by CTLA4-Ig inhibited both the in vitro proliferation (Fig. 3*A*) and the in vivo priming (Fig. 1*B*) of Ag-specific Th cells that is induced by each DC subset, whereas the blockade of the CD40-CD154 interaction inhibited only the CD8α^+ DC-induced differentiation of Th1 cells.

The current view of the function of CD40 in Th cell priming is that its ligation on DCs by CD154 on Ag-primed Th cells induces the maturation and activation of the DCs and promotes the secretion of proinflammatory cytokines such as IL-12 (22, 23), which is essential for the development of the Th1 response (24–27). With regard to the requirement for CD40-CD154 interactions for the development of appropriate immune responses, it is important to note that the T cell priming function of DCs is greatly influenced

by the activation status of DCs themselves after exposure to the Ag (14, 53). For example, human DCs infected with *Leishmania major* up-regulate their surface expression of costimulatory molecules, yet their IL-12 production is still dependent on CD40-CD154 interactions (29). In contrast, CD40-deficient murine myeloid DCs efficiently produce IL-12 upon exposure to *Propionibacterium acnes* (37). Similarly, although CD40 triggering is necessary for APCs to produce IL-12 in response to soluble protein Ags, LPS induces its production independently of CD40-CD154 interactions (54). It was recently shown that distinct DC subsets express different combinations of pattern-recognition receptors, such as Toll-like receptors (55, 56), that identify conserved molecular patterns shared by groups of microorganisms. Because signaling through pattern-recognition receptors can in itself lead to full activation of APCs, certain pathogens directly induce the maturation of and IL-12 production by specific DC subsets (57, 58), whereas others need additional factors to activate DCs. In the present study, we used DCs that had been cultured overnight and pulsed with soluble protein Ags. It has been reported that the CD8α^+ and CD8α^- DC subsets up-regulate their surface expression of MHC and costimulatory molecules and acquire T cell priming capacity through this procedure (14). As CD40 signaling is still required in this system for CD8α^+ DCs to produce IL-12 and IFN-γ (Fig. 6), the blockade of CD40-CD154 interactions results in the inhibition of the CD8α^+ DC-driven Th1 response. With respect to the unresponsiveness of CD8α^+ DCs to LPS stimulation (Fig. 6), it has been reported that DCs produce IL-12 only transiently after maturation and become refractory to LPS stimulation at later time points (59). In contrast to CD8α^+ DCs, CD8α^- DCs do not produce IL-12 regardless of CD40 triggering (Fig. 6), which means that Th2 priming is unaltered by the disruption of CD40-CD154 interactions. Notably, CD8α^- DCs do not even produce IL-10 in response to CD40 ligation (Fig. 6), which is reported to suppress IL-12 production by DCs (45). Our results demonstrate that, after optimal maturation, CD8α^- DCs can induce the Th2 response independently of CD40-CD154 interactions and that it is likely that this differential responsiveness of CD8α^+ and CD8α^- DCs to CD40 ligation underlies the distinct regulation of Th responses by each DC subset. Further studies focusing on signals downstream of the CD40 triggering in these DC subsets are warranted.

In summary, DCs are versatile APCs capable of discriminating potentially hazardous Ags from self or nontoxic environmental ones, and can mount an appropriate immune response depending on the nature of the Ag. Although our knowledge of DCs and their roles in the priming of Th cells has expanded, there are still many questions concerning the regulation of their functions. New insights into the complex mechanisms that govern the initiation of immunity by DCs will support the development of novel strategies for the treatment of autoimmune and allergic disorders, which are likely to be caused by the inappropriate regulation of DC functions.

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Limited Ability of Antigen-Specific Th1 Responses to Inhibit Th2 Cell Development In Vivo[1]

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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 CD8α^+ DCs induced a Th1 response and the production of IgG2a, whereas CD8α^- 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 CD8α^- DC transfer, CD8α^+ DCs failed to prime Th1 cells. In contrast, CD8α^- 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 CD8α^+ DCs in vitro, but the addition of IFN-γ did not effectively inhibit the potential of CD8α^- 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-γ 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-γ 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-γ-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-γ 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)[3] are APCs that initiate primary immune responses by activating naive Th cells (17, 18). In addition to presenting

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[3] Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand.

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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 CD8α homodimer and the type of Th response they induce. In vivo studies have shown that the adoptive transfer of Ag-pulsed CD8α^+ DCs triggers the development of Th1 cells, whereas CD8α^- 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 ($OVA_{323-339}$) in the context of the MHC class II molecule I-A^d (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-γ 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^{2+}-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 μM 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 CD8α^+ DCs were positively selected using anti-CD8 MicroBeads and autoMACS (Miltenyi Biotec). From the unselected fraction, CD8α^+ DCs were further depleted, and the CD8α^- DCs were positively enriched with anti-CD11c MicroBeads (Miltenyi Biotec). Flow cytometric analysis revealed that the purified fractions contained >96% CD8α^+CD11c$^+$ and CD8α^-CD11c$^+$ cells (data not shown).

Preparation of naive T cells from DO11.10 transgenic mice

CD4$^+$ cells were positively selected from spleen cells of DO11.10 transgenic mice using Dynabeads mouse CD4 and DETACHaBEAD mouse CD4 (Dynal Biotech) according to the manufacturer's instructions. From the enriched CD4$^+$ 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 μl of PBS were transferred i.v. into 8-wk-old BALB/c mice. A boost of 100 μg 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 administered, 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 μg/ml) in 48-well plates in X-VIVO 20 (BioWhittaker) supplemented with 0.5% mouse plasma and 50 μM 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^{high} CD44^{low} or CD62L^{low} CD44^{high} 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 μM 2-ME, and 20 ng/ml rGM-CSF, then cultured in 96-well, U-bottom plates (Falcon; BD Biosciences). rIFN-γ 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 μg/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 μg/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 μg/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-γ 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 CD8α^+ and CD8α^- DCs

We first evaluated the cytokine and Ab production profiles induced by the transfer of CD8α^+ and CD8α^- DCs. Thus, OVA-pulsed splenic CD8α^+ or CD8α^- 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 CD8α^+ DCs resulted in a much higher ratio of IFN-γ:IL-4 production by the splenocytes, whereas CD8α^- DC transfer induced Th2 responses with a low IFN-γ:IL-4 ratio (Fig. 1*A*). Moreover, OVA-specific IgG2a was only detected in the mice receiving CD8α^+ DCs, whereas OVA-specific IgE was only detected in mice given CD8α^- DCs (Fig. 1*B*). OVA-specific IgG1 was detected in mice that received either DC subset, although slightly higher levels were detected in mice that were given CD8α^- 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 CD8α^+ and CD8α^- DCs was confirmed using KLH as the Ag (Fig. 1*C*).

CD8α^+ 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 CD8α^- DCs, then 14 days later we administered OVA-pulsed CD8α^+ 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-γ and, instead, secreted a substantial amount of IL-4 (Fig. 2*A*). With regard to the serological response, OVA-specific IgG2a were not present in the sera of these

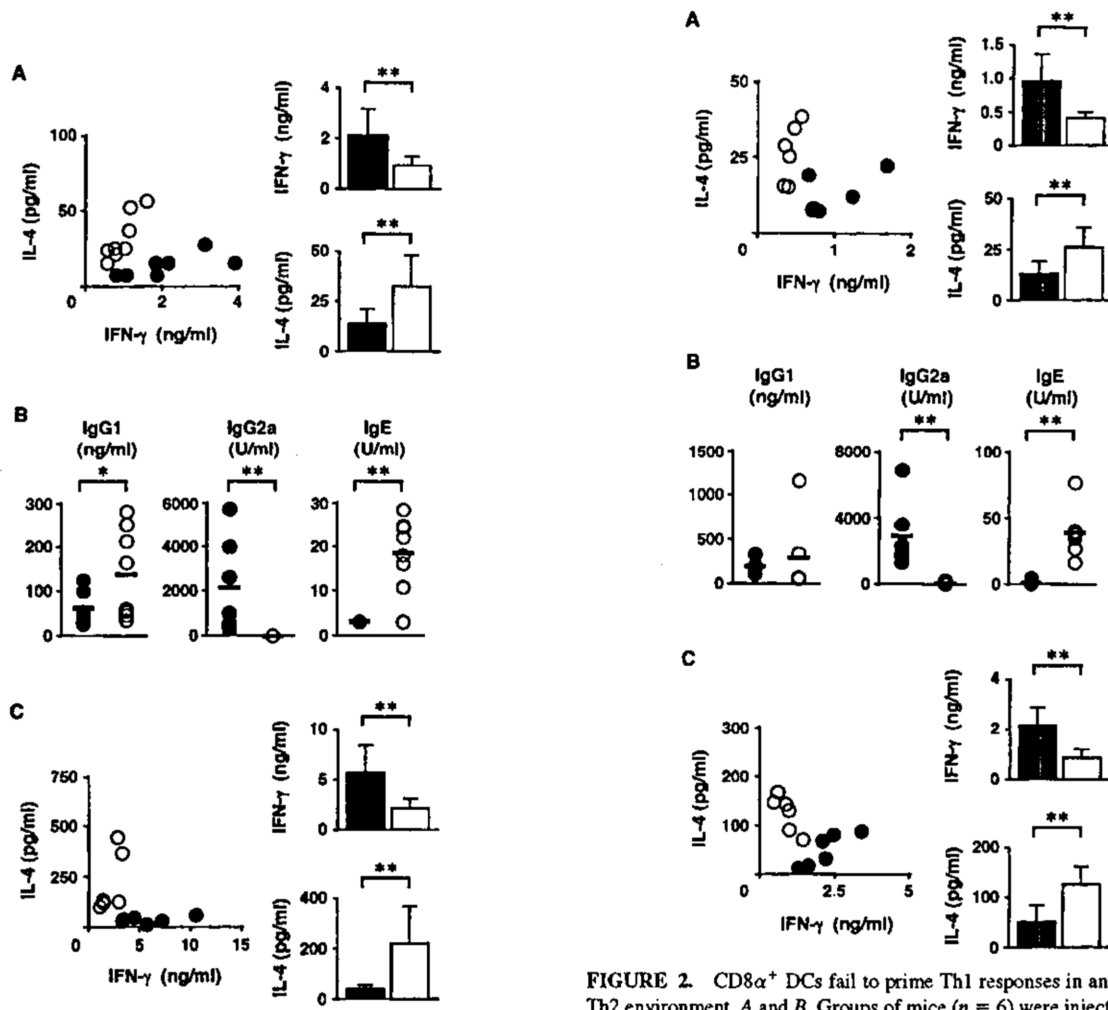

FIGURE 1. The transfer of Ag-pulsed CD8α^+ and CD8α^- DCs induces distinct types of Th and Ab responses. *A* and *B*, Groups of mice were given OVA-pulsed CD8α^+ ($n = 7$; ●) or CD8α^- ($n = 8$; ○) DCs and a boost of soluble OVA 7 days later. The mice were killed on day 14, and their sera and spleens 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 CD8α^+ DCs (●) or CD8α^- 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 ± 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. CD8α^+ DCs fail to prime Th1 responses in an Ag-specific Th2 environment. *A* and *B*, Groups of mice ($n = 6$) were injected with 2×10^5 OVA-pulsed CD8α^- DCs (○) or PBS (●) on day −14, and 2×10^5 OVA-pulsed CD8α^+ 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 2×10^5 KLH-pulsed CD8α^- DCs (○) or PBS (●) on day −14, and 2×10^5 KLH-pulsed CD8α^+ 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 ± 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. 2*B*). A similar phenomenon was observed when KLH was used as the Ag in place of OVA; namely, immunization with first KLH-pulsed CD8α^- DCs and then KLH-pulsed CD8α^+ DCs resulted in splenocytes that secreted high levels of IL-4, rather than IFN-γ (Fig. 2*C*).

CD8α^- 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 CD8α^+ DCs were administered before transfer of OVA-pulsed CD8α^- DCs. This resulted in splenocytes that produced IL-4 along with IFN-γ (Fig. 3*A*), namely, a mixed Th1/Th2 response. Moreover, both OVA-specific IgG2a and IgE were generated by this immunization regimen (Fig. 3*B*). When KLH was used as the Ag, both IL-4 and IFN-γ were again produced when the mice had been pretreated with CD8α^+ DCs (Fig. 3*C*).

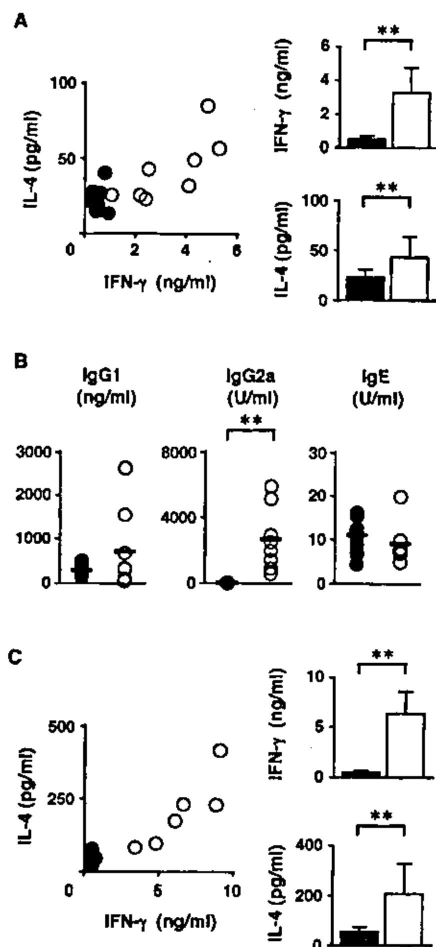

FIGURE 3. CD8α^- DCs can prime Th2 cells in the presence of Ag-specific Th1 cells. *A* and *B*, Groups of mice were injected with 2×10^5 OVA-pulsed CD8α^+ DCs ($n = 8$; ○) or PBS ($n = 11$; ●) on day −14, and 2×10^5 OVA-pulsed CD8α^- DCs were given on day 0. A boost of soluble OVA was given 7 days later, 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 2×10^5 KLH-pulsed CD8α^+ DCs (○) or PBS (●) on day −14, and 2×10^5 KLH-pulsed CD8α^- 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 ± 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).

CD8α^+ and CD8α^- DCs prime similar numbers of naive Th cells

We speculated that the opposite effects of CD8α^+ and CD8α^- 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 CD8α^- DCs prime many more Ag-specific Th cells than CD8α^+ DCs, this might leave fewer Ag-specific naive Th cells after the transfer of CD8α^- DCs, which might limit the subsequent Th1 priming induced by CD8α^+ 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. 4*A*, most DO11.10 Th cells remained CD62L^{high} CD44^{low} 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^{low} CD44^{high} phenotype (Fig. 4*A*). 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. 4*B*). However, the transfer of CD8α^- DCs did generate more activated Th cells (Fig. 4*C*). Thus, CD8α^+ and CD8α^- 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, CD8α^- DC-primed Th2 cells do proliferate more vigorously than CD8α^+ DC-primed Th1 cells.

Exogenous IL-4 abolishes the CD8α^+ DC-induced in vitro development of Th1 cells, but IFN-γ fails to suppress the development of Th2 cells induced by CD8α^- DCs

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

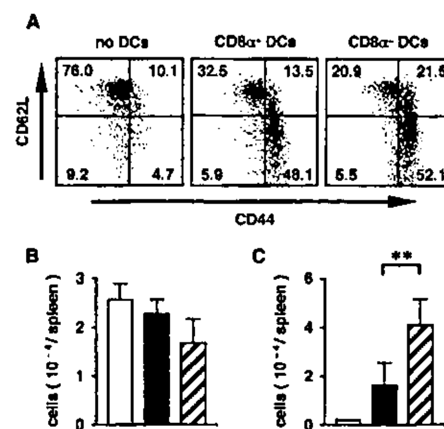

FIGURE 4. CD8α^+ and CD8α^- DCs prime similar numbers of naive Th cells. Naive DO11.10 Th cells were transferred i.v. into BALB/c mice 1 day before the administration of OVA-pulsed CD8α^+ DCs ($n = 4$; ■), CD8α^- DCs ($n = 6$; ▨), or PBS ($n = 3$; □). The mice were killed on day 14, and the phenotype of the KJ1-26$^+$ cells (*A*) and the absolute numbers of naive (*B*) or activated (*C*) KJ1-26$^+$ cells in spleens were determined. **, $p < 0.01$ (by Mann-Whitney *U* test).

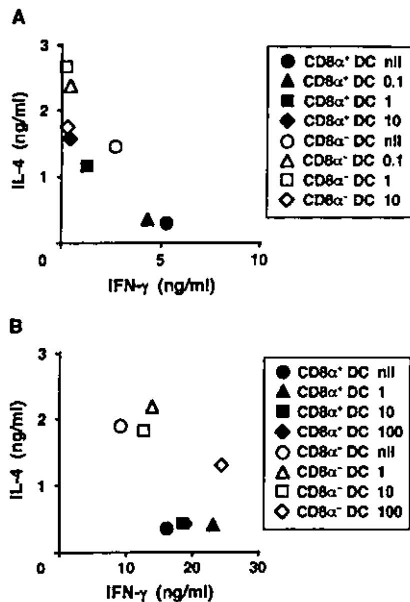

FIGURE 5. Effects of exogenous IL-4 and IFN-γ on in vitro DC-induced Th cell differentiation. Naive DO11.10 T cells were primed with OVA-pulsed CD8α^+ (●, ▲, ■, and ◆) or CD8α^- (○, △, □, and ◇) DCs. Graded doses of rIL-4 (*A*) or rIFN-γ (*B*; nanograms per milliliter) were added at the same time. The 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-γ and IL-4 to cultures of naive DO11.10 cells during their in vitro priming by CD8α^+ and CD8α^- DCs. In the absence of these exogenous cytokines, CD8α^+ DCs induced the development of Th1 cells, whereas CD8α^- 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 CD8α^+ DCs and induced the development of Th2 cells (Fig. 5*A*). In contrast, as much as 100 ng/ml rIFN-γ added to the priming culture only partially suppressed IL-4 production by CD8α^- DC-primed Th cells, although the production of IFN-γ was increased (Fig. 5*B*). These results suggest that the suppressive effects of IFN-γ on the induction of Th2 cells by CD8α^- DCs are limited, whereas IL-4 can overcome the Th1 priming signal provided by CD8α^+ 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 CD8α homodimer. There are about twice as many CD8α^- DCs as CD8α^+ 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 CD8α^+ nor CD8α^- 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 CD8α^+ DCs induced the development of Th1 responses and the production of IgG2a, whereas CD8α^- DCs primed a Th2 response and induced the production of IgE (Fig. 1). Because IFN-γ 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 CD8α^+ DCs, although a slightly higher level was induced by CD8α^- DCs (Fig. 1*B*). 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 CD8α^+ and CD8α^- 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 CD8α^- DCs, CD8α^+ 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 CD8α^+ DCs cannot effectively suppress subsequent priming of the Th2 response by CD8α^- DCs. Rather, in this situation the production of IL-4 increased along with that of IFN-γ, 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 CD8α^- DCs prime so many T cells that too few are left for CD8α^+ DCs to prime a sufficient response, because CD8α^+ and CD8α^- 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. 4*B*). However, there were twice as many CD8α^- DC-primed Th2 cells compared with CD8α^+ DC-primed Th1 cells (Fig. 4*C*), which means that CD8α^- DC-primed Th2 cells proliferate more vigorously than CD8α^+ DC-primed Th1 cells. These findings are in accordance with the observation of an in vitro study that shows that CD8α^+ DCs prime naive Th cells as well as CD8α^- 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 CD8α^+ DCs to inhibit subsequent Th2 priming by CD8α^- DCs could be due in part to the inability of CD8α^+ DCs to leave sufficient numbers of Th1 cells in vivo. However, we also found that exogenous IFN-γ did not efficiently inhibit the potential of CD8α^- DCs to prime IL-4-producing cells (Fig. 5*B*), whereas the addition of IL-4 abolished the Th1 priming potential of CD8α^+ DCs in vitro (Fig. 5*A*). 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-γ 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 β_2-chain on Th cells (38), hence blocking the IL-12 signal transduction pathway. In contrast, IFN-γ 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-γ treatment of early developing Th2 cells does not lead to their reduced IL-4 or increased IFN-γ production (38). Therefore, it is likely that exogenous IFN-γ could not overcome the Th2-inducing signal provided by CD8α^- DCs in the absence of IL-12 (Fig. 5*B*), whereas IL-4 by itself was sufficient to counteract the Th1-inducing signal provided by CD8α^+ DCs (Fig. 5*A*).

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 CD8α^- DCs could induce the production of IL-4 and IgE after Th1 priming with Ag-pulsed CD8α^+ 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 CD8α^+ DCs. There is the possibility that a single administration of CD8α^+ DCs could not induce a sufficiently strong Th1 response and that larger numbers or repetitive administration of CD8α^+ DCs may be required to prime enough Th1 cells to suppress the Th2 response induced by CD8α^- 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 CD8α^+ DCs induced a substantial amount of IFN-γ production in splenocyte cultures, which proves the efficacy of Th1 induction to a certain extent. The inhibition of Th1 priming by preimmunization with CD8α^- 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 CD8α^- DCs and by Th cells primed with CD8α^- DCs in vitro (data not shown). Thus, IL-10 produced by Th2 cells after an encounter with CD8α^+ 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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第45回日本臨床血液学会総会
教育講演6

先端医療開発に必要なGMP準拠細胞プロセッシング —Institutional GMP構築の必要性—

前 川 平

Key words : Cell therapy, Regenerative medicine, Gene therapy, Institutional GMP

I. はじめに

21世紀に入り爆発的スピードで展開しつつある再生医学の基礎研究と移植治療の進展を高所から鳥瞰すれば，「再生」と「移植」を融合させて構築した先端医療開発のプラットフォームからあたらしい治療法を創成し，その福音を一刻もはやく病める人々に還元することが社会的にも強く要請されていることは自明である。移植治療は臓器移植から細胞移植へと展開しつつあり，再生医学の基礎研究の成果は，少し前までは夢想だにしなかった事実をわれわれに教えてくれている。これらの研究成果を融合させ，わが国で開発したあたらしい治療法を世界に発信するためには，世界的なルールに基づいた細胞プロセッシング・システムを構築することが喫緊の課題である。

臨床試験研究には，科学的，倫理的に高い水準と信頼性が当然要求される。科学性はEvidence-Based Medicineで求められるものであり，倫理性はGCP（Good Clinical Practice：医薬品の臨床試験の実施基準），およびICH（日米欧医薬品規制ハーモナイゼーション国際会議）で担保される。信頼性はこれらを遵守することで保証される。GCPは，ニュールンベルグ綱領，ヘルシンキ宣言，リスボン宣言などを基盤として作り上げられてきたものであり，ICHは優れた医薬品のグローバルな研究開発の促進と承認審査資料の国際的ハーモナイゼーションの必要性を指摘したものである。ICH-GCPを形作るインフラストラクチャーの重要な部分を，GMP（Good Manufacturing Practice：医薬品の製造管理および品質管理に関する基準）およびGLP（Good Laboratory Practice：動物実験での標準操作手順と信頼性保証）が形成している。これらのことから，今後発展して行くと考えられる細胞治療，再生治療，遺伝子治療など，細胞自体を治療に応用しようとする探索的臨床試験研究（トランスレーショナル・リサーチ）にも，GMP準拠の細胞プロセッシングが不可欠であるとされている。もはや，わが国の治療開発研究だけが例外であることは許されないのが世界の趨勢である。細胞をもちいた治療法，すなわち再生治療や多くの遺伝子治療を開発しようとする場合，品質を保証するプロセッシングを受けた細胞を用いることで，探索的臨床試験研究の透明性と説明責任を担保でき，安全性と信頼性が高まり，ひいては患者さんにあたらしい治療法を早く提供できるという意味で医療倫理に直結する。

このように細胞治療（Cell Therapy）の開発には，細胞プロセッシング（Cell Processing）というヒト細胞の調製，培養，加工の工程が不可欠であり，その品質管理についてはGMPを遵守して行わなければならない。したがって，GMP準拠細胞プロセッシングがなければ，あたらしい細胞治療や再生治療の開発はできない。その根底には，安全な輸血療法に通じる考え方が脈々として流れている。

本稿では，第45回日本臨床血液学会総会教育講演で述べた事項を中心に，GMP準拠無菌的細胞プロセッシングについて，とくに先端医療開発，なかでも治療法として確立するかまだ判然としない（＝企業が本格的に参入することがまだ難しい）実験的治療の色彩の濃い，ヒト細胞をもちいた治療開発を行おうとする大学や研究所の目的に特化したGMP，すなわちinstitutional GMP構築の必要性を述べてみたい[1]。なお，具体的なハードに関してはすでに報告しているので参照されたい[2]。

II. わが国におけるGCPの制定と医薬品および治験薬GMP

医薬品GMPは医薬品の製造を厳密な管理のもとで行うための規範であり，米国，EU，日本，その他の国におけるGMPは，ほぼ同じ内容がそれぞれ特有のスタイルによりまとめられている。わが国においては，少なくと

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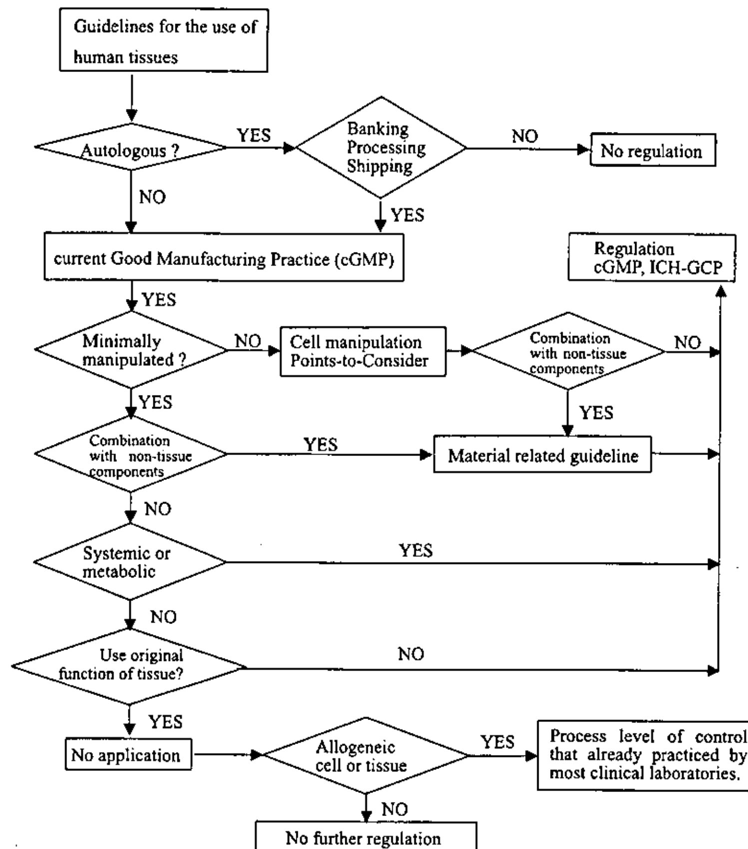

Fig. 1 Regulatory requirements associated with the proposed flow chart of cell and tissue based processing.

も次の3つの要件を満たすことが求められている。第1に「人為的な誤りを最小限にする」こと，第2に「医薬品に対する汚染および品質変化を防止すること」，第3に「高度な品質を保証するシステムを構築する」ことである。これは，現在，医療のリスクマネジメントにも求められていることに相通じるものであり，安全で効率的な輸血治療の実践と同様の事項が要求されているわけである。

医薬品にかかわる治験および市販後臨床試験を科学的かつ適正に実施するための基準として，平成9年3月27日厚生省令第28号で「医薬品の臨床試験の実施に関する省令」，いわゆるGCP省令が公布され，同年4月1日より実施された。GCP省令の第17条第1項において，「治験依頼者は，治験薬の品質の確保のために必要な構造設備を備え，かつ適切な製造管理および品質管理の方法が採られている製造所において製造された治験薬を実施医療期間に交付しなければならない」と定められている。これを受けて，治験薬を製造する際に遵守すべき「適切な製造管理および品質管理の方法の基準」(いわゆる治験薬GMPソフト) および「必要な構造設備の基準」(いわゆる治験薬GMPハード) として定められたものが治験薬GMPである。

A. ヒト細胞・組織医薬品GMPソフト

わが国におけるヒト細胞・組織医薬品GMPソフトは「細胞・組織医薬品等の取り扱いおよび使用に関する基本的考え方（薬務公報第1867号別添1，平成13年2月21日)」および「ヒト由来細胞・組織加工医薬品等の品質および安全性の確保に関する指針（同別添)」として示されている。これらは，平成8年度から10年度にかけて行われた厚生科学研究「組織細胞工学技術をもちいた医療材料・用具の有効性，安全性，品質評価に関する研究」(主任研究者　中村晃忠 国立医薬品食品衛生研究所・療品部長) の報告書[3]および国立医薬品食品衛生研究所報告書に早川尭夫氏が要約している論文[4]などを基に作成されたと思われる。報告の中で示されているフローチャートを改変して，Fig. 1に示す。

では，いったいどのような細胞操作が規制の対象となるのであろうか。米国FDAの考え方では，細胞操作が最低限度 (minimally manipulated) である場合は，とくに規制の対象とならないが，それを上回る有意な操作がおこなわれる場合 (more than minimally manipulated) は，規制の対象となり承認を必要とするとされている。"Minimally manipulated" の定義は，「操作がその組織の

本来の性質（＝再生や修復の機能をつかさどる能力に関する性質）を損なわない場合」とされている。たとえば，組織の切断，すりつぶし，洗浄，エチレンオキサイドやガンマー線による滅菌，細胞分離，凍結乾燥，凍結保存などは「最低限度の操作」とされている。わが国の実情を考慮して具体的な例をあげると，院内で行う（＝業として行わない）洗浄血小板や洗浄赤血球の作成，骨髄細胞や末梢血幹細胞の分離，凍結操作は「最低限度の操作」と考えられる。しかし，規制の対象にならないからと言って，いい加減で済まされるわけがない。国の承認は必要でなくても，院内で標準作業手順書（SOP: Standard Operating Procedure）を作成し，その操作方法などをしかるべき部署（たとえば輸血部）などにおいて管理するべきである。他方，"more than minimally manipulated"は，細胞を培養したり，骨髄細胞から血管内皮細胞を分離・培養したり，またサイトカインなどをもちいてある細胞分画を増幅させたり，樹状細胞や抗原特異的細胞障害性T細胞の培養，遺伝子導入，膵臓ランゲルハンス島細胞を分離して門脈経由で肝臓に移植したりする場合などがこの範疇に含まれる。当然，将来的にはES細胞をもちいた再生治療も含まれよう。cGMPあるいはcGTPの概念は，c（current）という言葉で表されるように，治療法開発の進展に伴い変わって行くものである。たとえば，CD34陽性細胞を分離して移植を行うことはmore than minimally manipulatedよりもminimally manipulatedとして考えられるようになってきている。

B．医薬品GMPおよび治験薬GMPからヒト細胞GMPへ

米国では細胞治療，再生治療および遺伝子治療等に用いる治療用細胞の作製は，2001年1月FDAの提言，cGTP (Good Tissue Practice："Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule"(cGTP) [21 CFR パート1271][5]に準拠して行われている。わが国では薬務公報第1867号及び厚生労働省告示第101号があり，平成15年7月から発効した改正薬事法・血液新法で"「生物由来製品」については，高度な製造工程管理を必要とすることを踏まえ，製造所における構造設備や製造管理・品質管理の方法（いわゆるGMP）に関し，通常の医薬品・医療機器等における製造基準に加え，厚生労働省令で定める付加的な基準に適合しなければならない"としている。これらに関する下位の法令は平成17年4月に公布予定とされている。その草案をみると，いわゆる錠剤などの医薬品GMPを基にしたもので，生物由来製品と言っても，血漿分画製剤や抗体治療薬などを念頭に置いたものである。錠剤や血漿分画製剤と，治療に用いようとする細胞では，そのプロセッシング方法は大きく異なる。細胞治療の開発にもちいる治療用細胞のプロセッシングに，錠剤などの医薬品GMPの規制を手直ししたとしても，そのまま適応することは難しいと懸念される。繰り返すが，錠剤，液体や蛋白質と，細胞のプロセッシングは当然異なる。

医薬品GMPでは平成11年4月に「薬局等構造設備規則」第8条の2として生物学的製剤などの製造所の製造設備に関する規定が，また平成13年4月には第8条の3としてヒト細胞・組織医薬品の製造所の構造設備に関する規定が追加されている。しかし，最近の細胞治療，再生治療，遺伝子治療などの進展を考慮したヒト細胞治療用GMPのハード面（施設の規格面）を指導する詳細なガイドラインがまだ制定されていないところに大きな問題がある。一方，米国FDAも1996年4月に発表された21 CFR 211のサブパートCのなかで施設のハード面を規定しているが，取り立てて詳細なものではない。"Any such building shall have adequate space for the orderly placement of equipment and materials to prevent mix-up between different components, drug products, and to prevent contamination"と述べるに留まっていたが，2002年9月，米国CBERからpreliminary concept paperとして，"Sterile Drug Products Produced by Aseptic Processing Draft"が発表され，現在パブリックコメントを聴取している最中である。序文には「Poor cGMP conditions at a manufacturing facility can ultimately pose a life threatening health risk to a patient」と明記されている。この中で，無菌的プロセッシング施設が具備すべき機能やその設計基準，および無菌的プロセッシング技術や管理の必要事項が具体的に示されている。これは主に医薬品の無菌的プロセッシングを念頭に置いているものであり，かなり厳しい内容になっているが，多くの事項は無菌的細胞プロセッシングにも適応可能であるように考慮されている。

III．Institutional GMPの必要性

米国の大学や先端医療開発センターで行われている細胞プロセッシングに，FDAはどのようにかかわっているのであろうか？　基礎研究の成果を臨床応用しようとする際，まず研究者や臨床研究医が臨床試験計画書を作成するとともに，それが細胞治療，再生治療，遺伝子治療などにかかわる場合には，細胞プロセッシングに必要な施設の詳細やバリデーション・マスタープラン，品質管理手順書，標準作業手順書（SOP）などGMP準拠細胞プロセッシングに必要な書類をFDAに提出する。FDAはこれらの研究計画に使用される治療用細胞を，IND (Investigational New Drug）として審査を開始するが，同時に施設の査察，およびGMP書類についても指導を行う。この際，FDAの係官は，錠剤などの医薬品GMPと細胞プロセッシングに必要なGMPとの差異を理解して

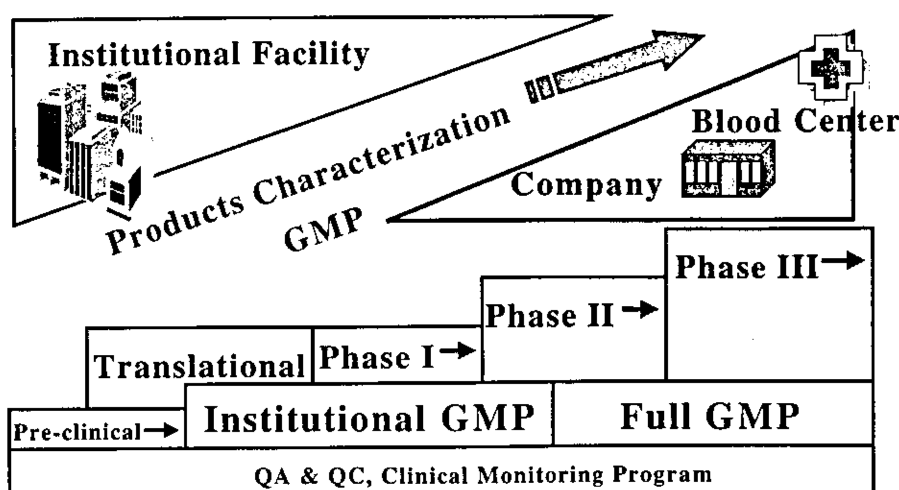

Fig. 2　Stepwise approach: regulatory requirements increase with product development. Institutional GMP should be established to advance translational research in academia.

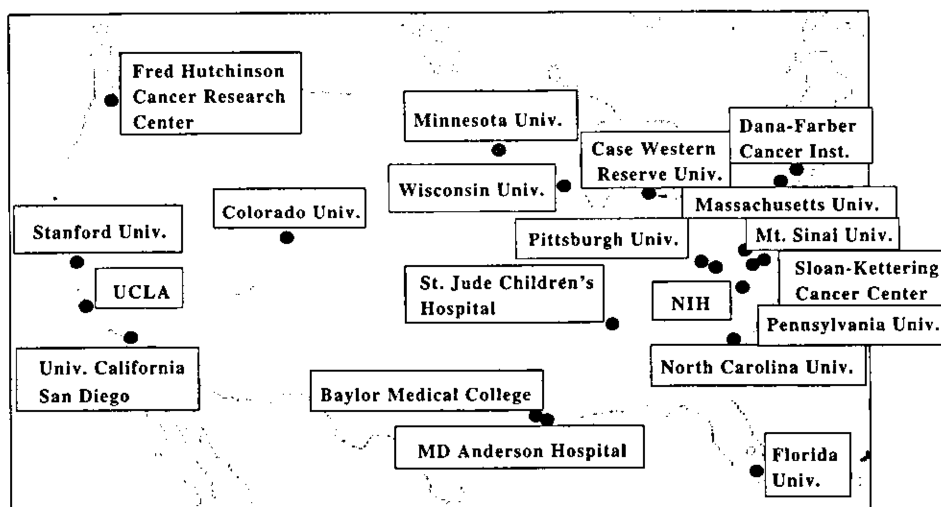

Fig. 3　Cell and molecular therapeutics facilities in USA

おり，企業などに要求される製品を作製するのに必要なGMP（=full GMP）の内容をもとにしつつも，大学などの実情を考慮した実験的治療開発に必要なGMP（=institutional GMP）の作成に協力を惜しまない。たとえば，膵臓ランゲルハンス島細胞移植に必要な細胞プロセッシングに関しては，FDAの係官が施設を査察するとともに，大学の人的余裕などを考慮し，研究者や臨床研究医とともにGMP構築に必要な書類を点検し，最低限必要な事項を示し，不備な点があれば改善するように指導を行っている。企業などに要求されるfull GMPと大学などに必要とされるinstitutional GMPの大きな違いは，前者では違反や不備があれば製造中止命令が出され罰則を伴うが，後者では改善するように勧告を行う，と言うことである。成文化されたinstitutional GMPがあるわけではないが，このように米国では大学などにおけるあたらしい治療法の開発を積極的に支援すると言うスタンスをとっている。わが国で，大学から厚生労働省にGMP査察を依頼した場合，おそらく人的資源の豊富な製薬企業で錠剤などを商品化するためのfull GMP，しかも細胞プロセッシングとは相容れない事項を要求してくるであろう。FDAが行っているようなinstitutional GMPを考慮した教育的指導が現時点では期待できないわが国では，研究者や臨床研究医自らがそのスタンダードを構築する必要がある。わが国においてinstitutional GMPを構築する必要があると主張する所以である（Fig. 2）。

「そのようなGMPの構築は大学で行うべきことでな

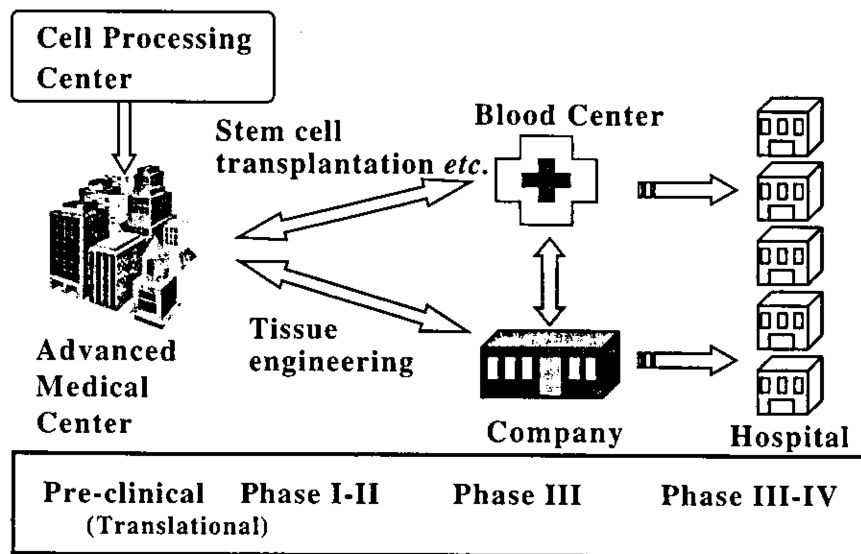

Fig. 4 Future prospects of cell and molecular therapeutic system in Japan

く，企業に任せておけば良い」と言う批判も耳にする。確かに，培養皮膚などすでに臨床応用されているような分野では企業の参入も期待できる。しかし，大学や先端医療センターで開発しようとしているのは，基礎研究の成果をもとにした，まだ実験的医療の段階のものがほとんどである。今後治療法として確立されるかどうか不透明であり，保険適応されるようになるかどうかなどまったく分からないのが現状であろう。このような段階の実験的医療開発の細胞プロセッシングにかかわるGMPの構築に，企業がリスクをとって積極的に参入することはきわめて困難である。ベンチャー企業を育成する土壌が未成熟なわが国においてはなおさらである。加えて，企業の中でも，細胞プロセッシングに必要なGMPのノウハウを確立しているところはきわめて少ない。このような現状では，大学や先端医療センターが主導するかたちで，研究者，臨床研究医，薬剤師，技師，GMPコンサルタント，企業の先端医療開発部門の研究者が一致協力して，世界的ルールにもとづいたものを構築して行く必要がある。

「全国各地の大学に細胞プロセッシングセンターを作る必要があるのか」と言う批判もある。確かに，莫大な費用がかかる細胞プロセッシングセンターを全国のすべての大学に建設する必要はないであろう。米国では，Fig. 3に示すように，先端医療開発を行っている大学や先端医療センターには，細胞プロセッシングセンターを併設しているところが多い。先端医療開発は集約すべきである。臨床応用可能であると言う目処がつけば，企業も参入が容易になるであろうし，また将来的に治療法として確立され，保険適用もされるようになれば，輸血製剤と同様に，全国に78箇所ある血液センターが細胞プロセッシングセンターの役割を担い，各病院へ供給するようになると考えている（Fig. 4）。

「細胞プロセッシングはサイエンスではない。大学や先端医療研究センターで行う必要はない」と言う意見もある。確かに，細胞プロセッシングは基礎研究などのピュアー・サイエンスではない。わが国では，基礎研究の成果を臨床応用しようとしても，そのインフラストラクチャーが不十分であるために欧米の後塵を拝している。基礎研究の成果を社会に還元する「実学」の必要性が認識されるようになり，そのインフラストラクチャーの一部を細胞プロセッシングが占める。科学知を社会に還元することの必要性を主張し，それを行うのが大学や先端医療研究センターであるべきと考えるならば，先端医療開発のための細胞プロセッシングをどこで行うべきかは明白であろう。現在，米国では，この細胞プロセッシングはレギュラトリー・サイエンスとして発展しつつある。

「細胞プロセッシングにGMPは本当に必要なのか」との疑問もよく聞く。すでに述べてきたことであるが，異なった観点からの例をあげる。食品衛生の分野ではHACCP（Hazard Analysis & Critical Control Point）と言う規制があり実施されている。牛乳などのパックには，HACCPに基づいて製品化されている旨が明記されているし，HACCPに基づいて製造されたものでなければ輸出できない。GMPはこのHACCPの根幹を形成している。某学会のランチョンセミナーの弁当で食中毒が発生したのは記憶にあたらしいが，これも衛生管理基準などGMPを形づくるルールに違反があったものと考えられる。

「Full GMPとinstitutional GMPのダブル・スタンダー

Table 1 Necessary requirements for the establishment of GMP cell processing at academic institution (seven elements for institutional GMP)

1. Facility design (completed)
2. Facility management (not yet completed, some parts are on going)
3. GMP documentation (still on going)
4. Educate and train GMP technicians (urgently needed and most important)
5. Close collaboration of clinicians, researchers, technicians, engineers and GMP consultants (necessary condition)
6. Close collaboration of academia, government, and company (necessary condition)
7. Financial support (necessary condition)

ドができることは好ましくない」との意見も聞く。行政側からすれば法令のダブル・スタンダードは好ましくないのは理解できる。米国でも institutional GMP が法令化されているわけではない。しかし，上述してきたように大学や先端医療開発センターでは institutional GMP が必要であり，法令でなく指針として示すべきと考える。

VI．今，必要なこと

著者が，わが国で最初の GMP 準拠細胞プロセッシングセンター計画（東大医科研）にかかわった平成 8 年には，その建設や設計のノウハウすらなかった。現在では，細胞プロセッシングセンター建設のハード面に関しては，条件が整ってきたと言える。その詳細は厚生労働科学研究費の報告書のなかで述べた[2]。施設建設のハード面はほぼ完成したものの，大学や先端医療センターで行う細胞プロセッシングの構築の中でとくに重要なことは，GMPに関する品質管理などの知識を持った技官の育成である（Table 1)。米国では，これらの技官を育成するためのトレーニング・コースがすでに設けられている。加えて，米国の先端医療開発センターに併設された細胞プロセッシングセンターでは，必ずと言ってよいほど，先端医療開発に関わる臨床研究医（MD）と研究者（PhD)，および専門知識をもった技官（PhD であることが多い）が一致協力してその運営にあたっている。

わが国においては，細胞治療・再生治療の先端医療開発に必須の細胞プロセッシングに関する詳細なガイドラインは提示されていない。この原因は，おそらく，実際に先端医療の開発に携わる医師や研究者がその重要性を認識してこなかったことにある。実際，先端医療の開発にかかわる臨床家や臨床研究医の多くは，たとえ細胞プロセッシングの重要性を認識していたとしても，これらは自らの守備範囲や研究範疇ではないし，論文などの業績にはならないとして蔑ろにしてきたと考えられる。あるいは，これらの細胞プロセッシングに関する事項や作業は外部に委託すれば良いと考え，自らこの分野に入ろうとしなかったと考えられる。これらの先端医療開発にかかわる臨床研究医も，今後，細胞治療・再生治療の開発に関する細胞プロセッシングの重要性を十分認識する必要があり，治療技術のひとつとして考えるようにならなければならない。しかも，細胞治療・再生治療の開発に必要な細胞プロセッシング技術の確立は，企業やクリーンルームの建設を請け負う建設会社のエンジニア，GMP や ISO の知識を有する専門家や薬剤師，薬学の専門家に任せておけば済むものではない。細胞治療や再生治療開発の細胞プロセッシング技術を我が国において根付かせるためには，これらの専門家とともに，実際の先端医療開発において診療にかかわる臨床研究医が自ら，GMP準拠細胞プロセッシングの必要性を認識し，異分野の研究者と「細胞プロセッシングの重要性」を共通言語として一致協力してルールづくりを行い，そこに厚生労働省の官僚の協力を得ることが必要である。臨床家や臨床研究医は，医学・医療の専門家としての立場から，エンジニアは空調システムなどの設計・建設の専門家としての立場から，薬剤師は薬学の専門家あるいは衛生管理の専門家としての立場から，厚生労働省の官僚は政策などの法制化の専門家としての立場から，すべての人々が「研究成果を社会に還元する」ことの重要性を認識して，同じテーブルについて議論しなければならない。

V．おわりに

わが国でも細胞治療や再生治療開発のためには，細胞プロセッシングセンターの必要性がようやく認識されてきたが，なかには単なる実験室のなかにクリーンベンチを置いて，それで大丈夫だとして同じインキュベーターで何人もの細胞を同時に培養したり，あるいは倫理委員会で承認されたから良しとして，ウシ胎児血清で培養した細胞を投与している施設がある。また，通常の実験室で研究者が作製したリポソームを投与している施設もある。現時点ではこれを規制する法律はわが国にはないが，