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VI. 研究成果の刊行物・印刷物

Differential Requirement for the CD40-CD154 Costimulatory Pathway during Th Cell Priming by CD8 α^+ and CD8 α^- Murine Dendritic Cell Subsets¹

Takahiro Yasumi,* Kenji Katamura,* Takakazu Yoshioka,* Taka-aki Meguro,* Ryuta Nishikomori,* Toshio Heike,* Manabu Inobe,[†] Shigeyuki Kon,[†] Toshimitsu Uede,[†] and Tatsutoshi Nakahata^{2*}

Dendritic cells (DCs) regulate the development of distinct Th populations and thereby provoke appropriate immune responses to various kinds of Ags. In the present work, we investigated the role CD40-CD154 interactions play during the process of Th cell priming by CD8 α^+ and CD8 α^- murine DC subsets, which have been reported to differently regulate the Th response. Adoptive transfer of Ag-pulsed CD8 α^+ DCs induced a Th1 response and the production of IgG2a Abs, whereas transfer of CD8 α^- DCs induced Th2 cells and IgE Abs in vivo. Induction of distinct Th populations by each DC subset was also confirmed in vitro. Although interruption of CD80/CD86-CD28 interactions inhibited Th cell priming by both DC subsets, disruption of CD40-CD154 interactions only inhibited the induction of the Th1 response by CD8 α^+ DCs in vivo. CD40-CD154 interactions were not required for the proliferation of Ag-specific naive Th cells stimulated by either DC subset, but were indispensable in the production of IL-12 from CD8 α^+ DCs and their induction of Th1 cells in vitro. Taken together, in our immunization model of Ag-pulsed DC transfer, CD40-CD154 interactions play an important role in the development of CD8 α^+ DC-driven Th1 responses but not CD8 α^- DC-driven Th2 responses to protein Ags. *The Journal of Immunology*, 2004, 172: 4826–4833.

Dendritic cells (DCs)³ are specialized APCs that activate naive T lymphocytes and initiate adaptive immunity (1, 2). In response to Ag presentation by DCs, naive Th cells differentiate into one of several functional subsets that differ in their cytokine secretion patterns and effector functions. Th1 cells secrete IFN- γ and promote cellular immunity by activating CTLs, NK cells, and macrophages. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, which induce Ig class switching to IgE and promote eosinophil-predominant inflammation. There are likely to be other Th subsets, such as Th0 and T regulatory cells, which produce various combinations of cytokines. The balance between these Th subsets determines the nature, strength, and duration of immune responses (3, 4).

The current understanding is that a series of defined signals must be delivered to naive Th cells to initiate effective immune responses. Along with the presentation of antigenic peptides to TCRs, appropriate costimulation must be provided for the activa-

tion and proliferation of naive Th cells. Furthermore, a large body of evidence suggests that DCs provide additional signals that instruct the functional differentiation of Th cells. These Th cell differentiation-regulating signals involve many factors, most of which are related to DCs (5, 6).

Two distinct DC subsets in the murine spleen have been characterized with regard to the expression of the CD8 α -chain (7, 8). In vitro studies suggest that CD8 α^+ DCs induce a limited T cell response and may play a role in the regulation of immune responses, whereas CD8 α^- DCs may be more stimulatory (9–11). In vivo studies have shown that injection of CD8 α^+ DCs triggers the development of Th1 cells whereas injection of CD8 α^- DCs induces a Th2-type response to soluble Ags (12, 13). It was later shown that neither CD8 α^+ nor CD8 α^- DCs can induce optimal T cell responses in their immature state and that their maturation is a prerequisite for both subsets to become potent activators of naive T cells (14). Moreover, recent studies revealed that CD8 α^+ DCs are responsible for maintaining peripheral tolerance under steady-state conditions (15, 16) but administration of an agonistic anti-CD40 Ab induces them to provoke a CTL response to tissue-associated Ags (16, 17). These studies clearly illustrate that the functional properties of each DC subset are not immutably fixed and that factors such as the maturation state of the DCs and the costimulatory molecules that they express contribute to the regulation of T cell priming.

Of the multiple costimulatory pathways that have been identified, CD40-CD154 interaction is thought to play a pivotal role in the process of T cell priming by DCs (18–20). CD154 is a member of the TNF family and is expressed primarily on activated Th cells. CD40, the receptor for CD154, is expressed on APCs such as B cells, macrophages, and DCs. CD40 ligation induces the maturation and activation of DCs (21) and promotes their secretion of cytokines such as IL-12 (22, 23), which is essential for the development of the Th1 response (24–27). In addition, exposure of DCs

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³ Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; hu-IgG, human IgG; ham-IgG, hamster IgG.

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 α^+ and CD8 α^- 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 α^+ and CD8 α^- 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 α^+ DCs but had little effect on the Th2 response induced by CD8 α^- DCs. Our results indicate that CD40-CD154 interactions play an important role in the development of CD8 α^+ DC-driven Th1 responses but not in CD8 α^- 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-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 Ca²⁺-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 μ M 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 α^+ DCs were positively selected using anti-CD8 MicroBeads and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). From the negative fraction, CD8 α^+ DCs were further depleted and CD8 α^- DCs were positively enriched with anti-CD11c MicroBeads (Miltenyi Biotec). Flow cytometric analysis revealed that each purified fraction contained >96% CD8 α^+ CD11c⁺ and CD8 α^- 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 \times 10⁵ 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 μ g 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 μ g 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 \times 10⁷ 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 (10⁶ cells/well) and 30 Gy-irradiated BALB/c splenocytes (10⁶ cells/well) were cocultured in 48-well plates in X-VIVO 20 (BioWhittaker) supplemented with 0.5% mouse plasma and 50 μ M 2-ME with or without KLH (100 μ g/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 \times 10³ cells/well) and naive DO11.10 T cells (5 \times 10⁴ cells/well) were suspended in X-VIVO 15 supplemented with 0.5% mouse plasma, 50 μ M 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 \times 10⁵ cells/well) from C57BL/6 mice were stimulated with graded numbers of BALB/c DCs. [*methyl*-³H]Thymidine (0.5 μ Ci/well; Moravak 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 \times 10⁴ cells/well) and naive DO11.10 T cells (2 \times 10⁵ 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 μ g/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 \times 10⁵ cells/well) were restimulated in a 96-well U-bottom plate with 30 Gy-irradiated BALB/c splenocytes (2 \times 10⁵ cells/well) and OVA (100 μ g/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 Ca²⁺-free HBSS containing 10 mM EDTA and labeled with PE-conjugated anti-CD28 (37.51; eBioscience), anti-CD40, anti-CD80, anti-CD86, anti-CD152 (UC10-4B9; eBioscience) or anti-CD154 (MR1; eBioscience) 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 μ M 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 μ g/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 μ g/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 μ g/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 CD8 α^+ 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 CD8 α^+ and CD8 α^- 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 CD8 α^+ DCs induced a much higher level of IFN- γ production, whereas CD8 α^- DCs preferentially induced the production of IL-4 and IL-10 (Fig. 1A). 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 CD8 α^+ and CD8 α^- DCs, respectively (Fig. 1B). Administration of CD40-Ig also inhibited the IFN- γ production of the splenic CD4⁺ cells induced by CD8 α^+ DC-transfer (Fig. 1C). In contrast, the IL-4 and IL-10 production by CD8 α^- DC-transferred mice

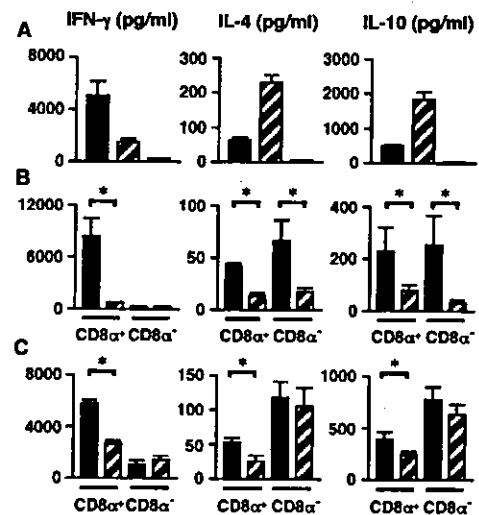


FIGURE 1. Effect of CTLA4-Ig and CD40-Ig on CD8 α^+ DC-induced and CD8 α^- DC-induced Th responses. *A*, Groups of BALB/c mice ($n = 3$) were injected with KLH-pulsed CD8 α^+ DCs (■), CD8 α^- DCs (□), or PBS (□). Fourteen days later, CD4⁺ cells were positively selected from the pooled splenocytes of 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 CD8 α^+ DCs or CD8 α^- 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 \pm 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. 1C). These results indicate that while CD80/CD86-CD28 interactions are required for the Th cell priming by both CD8 α^+ and CD8 α^- DCs, CD40-CD154 interactions are only required for the induction of the Th1 response by CD8 α^+ 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 CD8 α^+ and CD8 α^- 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. 2A, OVA-specific IgG2a was detected specifically in the mice that had received the CD8 α^+ DCs whereas OVA-specific IgE was detected in mice given CD8 α^- 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 μ g/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. 2A). 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

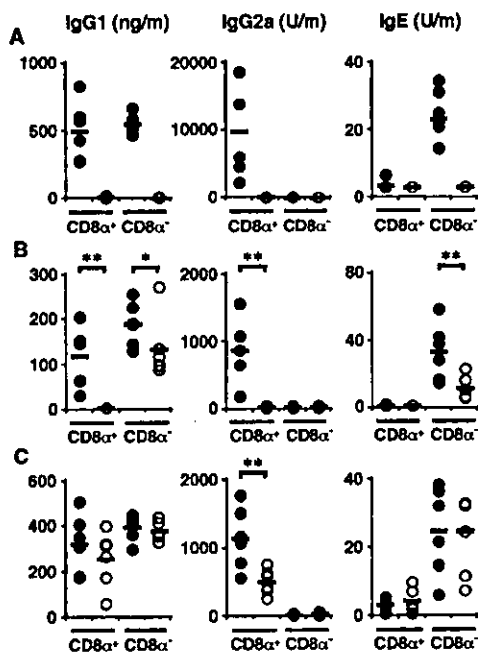


FIGURE 2. Effect of CTLA4-Ig and CD40-Ig on CD8 α^+ DC-induced and CD8 α^- DC-induced Ab responses. *A*, Groups of mice ($n = 6-7$ per group) received OVA-pulsed CD8 α^+ or CD8 α^- DCs and a boost of soluble OVA (●) or PBS (○) 14 days later. Sera were collected 7 days later and assayed for OVA-specific Ab isotype titers by ELISA. *B* and *C*, Groups of mice ($n = 5-6$ per group) received OVA-pulsed CD8 α^+ or CD8 α^- 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.

CTLA4-Ig (Fig. 2*B*) but CD40-Ig only inhibited the production of IgG2a induced by the CD8 α^+ DCs (Fig. 2*C*). CD40-Ig did not affect the levels of IgG1 induced by the CD8 α^+ DCs or the IgG1 and IgE Abs induced by CD8 α^- 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 CD8 α^+ DCs in vivo.

CD40-CD154 interactions are not required for DC-induced proliferation of Th cells but are indispensable for effective Th1 priming by CD8 α^+ 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 CD8 α^+

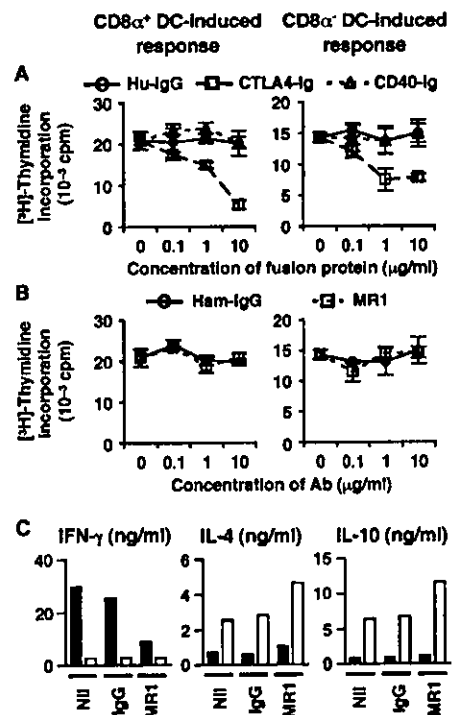


FIGURE 3. The CD40-CD154 interaction is not required for the proliferation of naive Th cells, but is indispensable for the induction of Th1 cells by CD8 α^+ DCs in vitro. *A* and *B*, Naive DO11.10 CD4 $^+$ T cells were cultured for 72 h with OVA-pulsed CD8 α^+ (*left*) or CD8 α^- (*right*) DCs. CTLA4-Ig, 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 CD8 α^+ (■) or CD8 α^- (□) 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.

(Fig. 3*A, left*) and CD8 α^- 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, CD8 α^- DC-primed T cells primarily produced IFN- γ whereas IL-4 and IL-10 were preferentially produced by CD8 α^- DC-primed T cells (Fig. 3*C*). However, when the MR1 Ab was present during the priming, the production of IFN- γ by CD8 α^+ DC-primed T cells was reduced (Fig. 3*C*). In contrast, the IL-4 and IL-10 production by CD8 α^- 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 CD8 α^+ DC-induced differentiation of Th1 cells.

CD8 α^+ and CD8 α^- DCs express similar levels of costimulatory molecules

We next analyzed the Ag-uptake potential and the surface expression of costimulatory molecules on CD8 α^+ and CD8 α^- 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. 4A). About one-half of the CD8 α^- DCs expressed CD4, whereas the CD8 α^+ DCs did not (Fig. 4A). In addition, both DC subsets induced a vigorous allogeneic MLR (Fig. 4B), although CD8 α^- DCs induced slightly higher response (9).

CD8 α^+ and CD8 α^- 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. 5A). Moreover, similar levels of CD154 expression were induced on Th cells by either DC subset (Fig. 5B). 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 CD8 α^+ DCs after CD40 triggering

It has been suggested that DC-derived IL-12 plays a crucial role in determining the Th1/Th2-promoting capacity of the CD8 α^+ and CD8 α^- DCs, and that IFN- γ promotes a Th1 response by inducing IL-12 production from CD8 α^+ DCs whereas IL-10 promotes a Th2 response by inhibiting the IL-12 production by CD8 α^- DCs

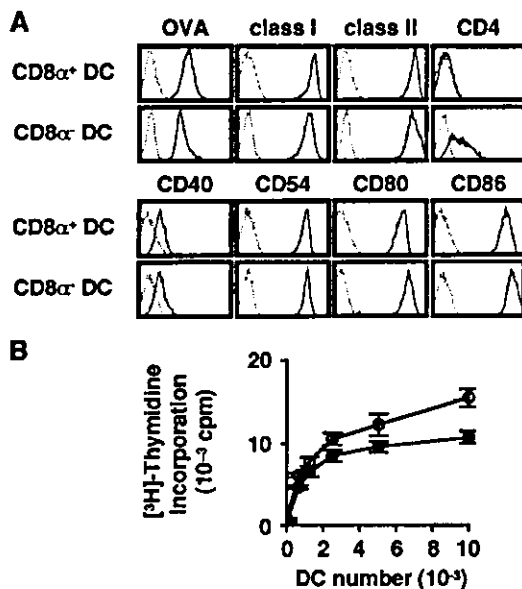


FIGURE 4. CD8 α^+ and CD8 α^- DCs express similar levels of costimulatory molecules. *A*, CD8 α^+ and CD8 α^- DCs were pulsed with FITC-OVA and analyzed for Ag-uptake potential. Their surface expression of CD4, CD40, CD54, CD80, CD86, MHC class I and class II molecules was also evaluated. The dotted lines represent DCs pulsed with unlabeled OVA or stained with isotype controls. *B*, Graded numbers of CD8 α^+ (●) and CD8 α^- DCs (○) were cultured with C57BL/6 splenic CD4⁺ cells for 72 h. Proliferation was measured by pulsing the cultures with [³H]thymidine for the last 8 h. The data shown are representative of at least three independent experiments, all with similar results.

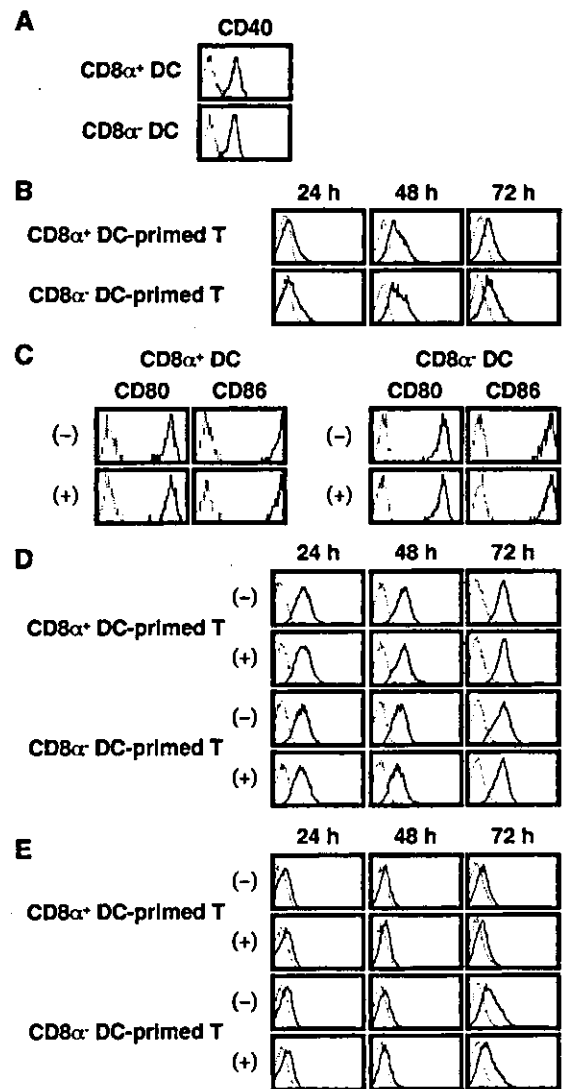


FIGURE 5. CD8 α^+ and CD8 α^- DCs express similar levels of CD40 after interaction with Th cells and induce similar levels of CD154 expression. *A*, OVA-pulsed CD8 α^+ and CD8 α^- DCs were cultured with naive DO11.10 CD4⁺ T cells and surface expression of CD40 was analyzed 24 h later. *B*, Naive DO11.10 T cells were primed with OVA-pulsed CD8 α^+ or CD8 α^- 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.

(45). Consequently, we analyzed the cytokine production of CD8 α^+ and CD8 α^- DCs when they were cultured in the presence of an agonistic anti-CD40 mAb. CD8 α^+ DCs produced IL-12 and IFN- γ after this CD40 triggering (Fig. 6). However, the CD8 α^+ DCs also produced substantial amounts of IL-10 regardless of CD40 signaling (Fig. 6). In contrast, CD8 α^- 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

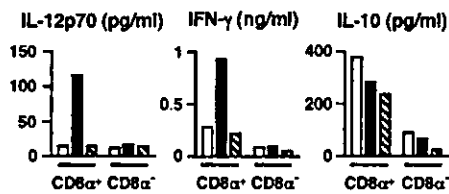


FIGURE 6. Selective production of cytokines by CD8 α^+ DCs after CD40 triggering. Sorted DC subsets were left untreated (\square) or stimulated either with an agonistic anti-CD40 mAb (\blacksquare) or LPS (▨). The culture supernatants were harvested 24 h later and assayed for cytokine concentrations 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 CD8 α^+ DCs triggers the development of Th1 cells, whereas injection of CD8 α^- 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. 2A, CD8 α^+ and CD8 α^- 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 CD8 α^+ DCs induced much higher levels of IFN- γ , whereas IL-4 and IL-10 production was preferentially induced by CD8 α^- DCs (Fig. 1A). 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. 2A). 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. 3C). Notably, the distinct effect of these subsets on Th cell differentiation, in particular the induction of Th2 cells by CD8 α^- 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 CD8 α^+ and CD8 α^- 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 CD8 α^+ DCs but not for the induction of a Th2 response by CD8 α^- DCs *in vivo* (Fig. 1C). 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 CD8 α^+ DCs (Fig. 2C). CD40-Ig had no effect on the Ab profile generated by CD8 α^- DCs (Fig. 2C). In accordance with these *in vivo* results, CD40-CD154 blockade inhibited the Th1 differentiation of Ag-specific naive Th cells stimulated by CD8 α^+ DCs *in vitro* (Fig. 3C). 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 CD8 α^+ DC-induced differentiation of Th1 cells but not for the activation of naive Th cells or the CD8 α^- 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 CD8 α^+ and CD8 α^- DCs expressed similar levels of CD40 before (Fig. 4A) and 24 h after (Fig. 5A) 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 CD8 α^+ and CD8 α^- DC subsets induced similar levels of CD154 expression on naive Th cells (Fig. 5B) 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 up-regulate 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 CD8 α^+ and CD8 α^- DCs already express high levels of CD80 and CD86 (Fig. 4A), 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. 3A) and the *in vivo* priming (Fig. 1B) of Ag-specific Th cells that is induced by each DC subset, whereas the blockade of the CD40-CD154 interaction inhibited only the CD8 α^+ 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 CD8 α^+ and CD8 α^- 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 CD8 α^+ DCs to produce IL-12 and IFN- γ (Fig. 6), the blockade of CD40-CD154 interactions results in the inhibition of the CD8 α^+ DC-driven Th1 response. With respect to the unresponsiveness of CD8 α^+ 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 CD8 α^+ DCs, CD8 α^- 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, CD8 α^- 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, CD8 α^- DCs can induce the Th2 response independently of CD40-CD154 interactions and that it is likely that this differential responsiveness of CD8 α^+ and CD8 α^- 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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Development of primitive and definitive hematopoiesis from non-human primate embryonic stem cells in vitro

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Summary

Although information about the development of primitive and definitive hematopoiesis has been elucidated in murine embryos and embryonic stem (ES) cells, there have been few *in vitro* studies of these processes in primates. In this study, we investigated hematopoietic differentiation from cynomolgus monkey ES cells grown on OP9, a stromal cell line deficient in macrophage colony-stimulating factor. Primitive erythrocytes (EryP) and definitive erythrocytes (EryD) developed sequentially from ES cells in the culture system; this was confirmed by immunostaining and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of embryonic, fetal and adult globin genes. EryP were detected on day 8 without exogenous erythropoietin (EPO), whereas EryD appeared on day 16 and had an indispensable requirement for exogenous EPO. RT-PCR analysis of the cultures revealed a sequential expression of genes associated with primitive and definitive

hematopoietic development that was equivalent to that seen during primate ontogeny *in vivo*. Vascular endothelial growth factor (VEGF) increased, in a dose-dependent manner, not only the number of floating hematopoietic cells, but also the number of adherent hematopoietic cell clusters containing CD34-positive immature progenitors. In colony assays, exogenous VEGF also had a dose-dependent stimulatory effect on the generation of primitive erythroid colonies. More efficient primitive and definitive erythropoiesis was induced by re-plating sorted CD34-positive cells. Thus, this system reproduces early hematopoietic development *in vitro* and can serve as a model for analyzing the mechanisms of hematopoietic development in primates.

Key words: ES cells, Primate, Primitive hematopoiesis, Definitive hematopoiesis

Introduction

During murine embryogenesis, primitive (embryonic) hematopoiesis is first detected in blood islands of the yolk sac at approximately 7.5 days postcoitum (dpc). It is followed by definitive (fetal and adult) hematopoiesis in aorta-gonad-mesonephros regions, which shifts to the fetal liver, spleen and bone marrow (Dzierzak and Medvinsky, 1995; Xu et al., 2001). Primitive hematopoiesis generates mainly nucleated primitive erythrocytes (EryP), whereas definitive hematopoiesis generates all hematopoietic lineages, including nucleated and enucleated definitive erythrocytes (EryD) and hematopoietic stem cells with a long-term repopulating activity. Pluripotent murine embryonic stem (ES) cells have been used as a powerful tool for studying the mechanisms of hematopoietic development in various culture conditions (Doestchmann et al., 1985; Wiles and Keller, 1991; Keller et al., 1993; Nakano et al., 1996; Nishikawa et al., 1998). Among these conditions, co-

culture with OP9 stromal cells, which lack functional macrophage colony-stimulating factor (M-CSF; CSF1 – Mouse Genome Informatics) (Yoshida et al., 1990), can induce primitive and definitive hematopoietic development in murine ES cells *in vitro* in a pattern similar to that seen in murine ontogeny (Nakano et al., 1996).

Recently, it has been reported that primate hematopoiesis occurs in a manner similar to that of mice, based on immunohistochemical studies of human embryos (Tavian et al., 1996; Tavian et al., 1999). In the erythrocytes of primates, the embryonic (ϵ and ζ), fetal (γ) and adult (β and α) globin genes are expressed sequentially during development, although small amounts of fetal and adult globin chains are detected even during primitive hematopoiesis (Johnson et al., 2000; Stamatoyannopoulos et al., 2001). Concomitant switches in the α cluster (replacement of the ζ globin gene by the α) and β cluster (replacement of the ϵ globin gene by the γ , and

replacement of the γ globin gene by the β) occur during development, and coincide with the transition from yolk sac to fetal liver, and finally to bone marrow hematopoiesis.

The hematopoietic development of primates remains to be elucidated, in part because of the ethical restrictions on experiments using their embryos. Old World monkeys, such as the cynomolgus monkey (*Macaca fascicularis*), are widely used for medical research (Hanazono et al., 2000) and have globin gene expression that is similar to that of humans (Johnson et al., 2000). Therefore, their ES cells might be used as a model for elucidating primate hematopoietic development. Recently primate ES cell lines were established (Thomson et al., 1995; Thomson et al., 1996; Thomson et al., 1998; Suemori et al., 2001), and hematopoietic differentiation from primate ES cells was also induced successfully in vitro (Kaufman et al., 2001; Li et al., 2001; Lu et al., 2002; Chadwick et al., 2003). However, compared with the murine system, little work has been done to precisely analyze primitive and definitive hematopoietic development.

To address this problem, we induced hematopoietic differentiation in cynomolgus monkey ES cells by co-culture with OP9 stromal cells. This is the first report to demonstrate that primitive hematopoiesis and its transition to definitive hematopoiesis can be induced from primate ES cells in vitro.

Materials and methods

Maintenance of cynomolgus monkey ES cell line

The ES cell line CMK6 was established from cynomolgus monkey blastocysts produced by in vitro fertilization or intracytoplasmic sperm injection and maintained as previously described (Suemori et al., 2001). Briefly, cells were transferred onto a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (MEF) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (Sigma, St Louis, MO), supplemented with 20% KnockOut SR serum replacer (Gibco BRL, Grand Island, NY), 100 μ M 2-mercaptoethanol (2ME) (Nakalai Tesque, Kyoto, Japan), 2 mM L-glutamine and 1% nonessential amino acids (Gibco BRL). ES cells were passaged every 3 days to maintain their undifferentiated state, and were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

Cytokines and growth factors

Recombinant human granulocyte colony-stimulating factor (G-CSF; CSF2 – Human Gene Nomenclature Database), EPO, interleukin 3 (IL3), stem cell factor (SCF) and thrombopoietin (THPO) were kindly provided by Kirin Brewery (Tokyo, Japan). Recombinant human VEGF, basic fibroblast growth factor (bFGF), and bone morphogenetic protein (BMP) 4 were all purchased from R&D Systems (Minneapolis, Minnesota).

Antibodies

The primary antibodies used in this study were: mouse anti-human CD41 (clone 5B12) and CD45 (clone T16) antibodies (Dako, Kyoto, Japan); mouse anti-human CD34 (clone 563) antibody (Becton-Dickinson, San Jose, CA); KIT antibody (Nichirei, Tokyo, Japan); rabbit anti-human hemoglobin (Hb) polyclonal antibody (Cappel, Aurora, Ohio); and sheep anti-human fetal hemoglobin (HbF) polyclonal antibody (Bethyl, Montgomery, TX). The mouse anti-human embryonic ϵ -globin (HbEmb) monoclonal antibody, and anti-human fetal liver kinase (FLK) 1 monoclonal antibody were used as described previously (Luo et al., 1999; Sawano et al., 2001). Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-sheep IgG, fluorescein isothiocyanate (FITC)-conjugated donkey anti-

rabbit IgG and alkaline phosphatase (ALP)-conjugated donkey anti-mouse IgG (all purchased from Jackson ImmunoResearch Laboratories, West Grove, PA), and allophycocyanin (APC)-conjugated goat anti-mouse IgG (Becton-Dickinson), were used as secondary antibodies.

In vitro hematopoietic differentiation from ES cells

OP9 stromal cells were kindly provided by Dr Hiroaki Kodama, and were maintained in α MEM (Gibco BRL) supplemented with 20% fetal calf serum (FCS) (EQUITECH-BIO, Kerrville, TX). Trypsin-treated ES cells (4×10^3 cells/well) were transferred onto confluent OP9 stromal cells in α MEM supplemented with 10% FCS and 50 μ M 2ME, in the presence or absence of VEGF, bFGF or BMP4. On day 6 of differentiation, the induced cells were harvested with cell dissociation buffer (Invitrogen, Carlsbad, CA). Then the cells were filtered through a 70 μ m nylon cell strainer (Falcon, Lincoln Park, NJ), and 1×10^5 cells/well were transferred onto fresh confluent OP9 cells in 6-well plates and cultured in α MEM supplemented with 10% FCS and 50 μ M 2ME, in the presence or absence of EPO (10 U/ml). The medium was changed every 2 or 3 days during the induction of differentiation. Adherent hematopoietic cell clusters, which consisted of more than 20 round blast-like cells, were counted using an inverted microscope. The same series of experiments was performed at least three times.

Staining

For cytochemical staining, the floating cells were centrifuged onto glass slides and analyzed by microscopy after May-Giemsa or myeloperoxidase staining.

For immunostaining, floating cells spun onto glass slides were fixed in 4% paraformaldehyde and permeabilized with phosphate buffered saline (PBS) containing 5% skim milk (Becton-Dickinson) and 0.1% Triton X-100 for 30 minutes. The cells were then incubated with primary antibodies overnight, washed three times with PBS containing 5% skim milk, and then incubated with FITC- or Cy3-conjugated secondary antibodies for 30 minutes. Nuclei were labeled with Hoechst 33342 (Molecular Probes, Eugene, Oregon). The cells were then washed three times with PBS and observed by fluorescence microscopy (Olympus, Tokyo, Japan). In the human erythroblastic cell line K562, which is known to express ϵ , ζ , γ and α globins (Rutherford et al., 1981), all erythroid cells were positive for Hb, HbF and HbEmb. In adult cynomolgus bone marrow, all erythrocytes were positive for Hb and a few were positive for HbF, whereas HbEmb-positive erythrocytes were rarely detected (data not shown). The adherent cells were fixed and incubated with primary and ALP-conjugated secondary antibodies, as described above, and positive cells detected using a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). Endogenous ALP activity was blocked by 2 mM levamisole (Wako, Osaka, Japan).

Methylcellulose colony forming assays

The medium was replaced with a fresh semisolid medium consisting of α MEM, 0.9% methylcellulose, 30% FCS, 10% bovine serum albumin and 50 μ M 2ME, and a mixture of human G-CSF 10 ng/ml, EPO 2 U/ml, IL3 20 ng/ml, SCF 100 ng/ml and THPO 10 ng/ml, as previously reported (Sui et al., 1995). All cultures were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air. Seven days later, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, washed twice with PBS, and processed for May-Giemsa staining, immunostaining and RT-PCR analysis. Colonies (≥ 50 cells) were counted using an inverted microscope according to the criteria previously reported (Nakahata and Ogawa, 1982; Tajima et al., 1996).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by using TRIzol (Invitrogen) according to the manufacturer's protocol. Each total RNA sample was then reverse-

transcribed using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified in a final volume of 20 μ l PCR buffer containing 2.5 mM MgCl₂ and 250 μ M dNTP, using Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). As cynomolgus monkey-specific sequences were unavailable, we employed those of corresponding human or other Old World monkey genes to design our PCR primers, based on the generally close homology between human and cynomolgus monkey gene sequences. The EPO receptor (EPOR) and α -fetoprotein (AFP)-specific primers have been described previously (Schuldiner et al., 2000; Yokomizo et al., 2002). Samples were initially denatured at 94°C for 5 minutes, followed by amplification rounds consisting of 94°C for 1 minute (denaturing), 57-66°C for 1 minute (annealing) and 72°C for 1 minute (extension), and then a final extension at 94°C for 7 minutes. The oligonucleotide primers were:

ϵ -globin (450 bp), 5'-TGCATTTACTGCTGAGGAGA-3' (sense) and 5'-AAGAGAAGCTCAGTGGTACTT-3' (antisense);
 γ -globin (524 bp), 5'-AGACGCCATGGGTCATTTTACA-3' (sense) 5'-GCCTATGGTTGAAAGCTCTGIAT-3' (antisense);
 β -globin (196 bp), 5'-GGGACGGCTGCTGGTGGTCTAC-3' (sense) and 5'-CGTGTGGTGTGTGTGGTGG-3' (antisense);
 ζ -globin (223 bp), 5'-TTCCTCAGCCACCCGACAG-3' (sense) and 5'-AGCAGGCAGTGGGACAGGAG-3' (antisense);
 α -globin (152 bp), 5'-TGCACGGCACAAGCTTCGG-3' (sense) and 5'-GCACGGTGTACAGAAAGCCAG-3' (antisense);
 EPOR (183 bp), 5'-TGGTATCTGACTCTGGCATCTC-3' (sense) and 5'-TCCCTGATCACTGCAGCC-3' (antisense);
 Brachyury (253 bp), 5'-AAGGTGGATCTTCAGGTAGC-3' (sense) and 5'-CATCTATTGGTGAAGTCC-3' (antisense);
 FLK1 (239 bp), 5'-AAAACCTTTTGTGCTTTTGG-3' (sense) and 5'-GAAATGGGATTGGTAAGGATG-3' (antisense);
 SCL (185 bp), 5'-TCTCGGCAGCGGGTCTTTG-3' (sense) and 5'-AAGCCCCGTTTACAT TCTGC-3' (antisense);
 LMO2 (213 bp), 5'-CTGATGCTTGGCCTTCTCTCC-3' (sense) and 5'-GGCCCAGTTTGTAGTAGAGGC-3' (antisense);
 MYB (307 bp), 5'-CACGCTGGGCTGTCATCAAC-3' (sense) and 5'-GCATGGCTCTTCGTGTTATAGC-3' (antisense);
 GATA-2 (303 bp), 5'-TGGCGCACAACACTACATGGAAC-3' (sense) and 5'-GAGGGGTGCAGTGGCGTCTT-3' (antisense);

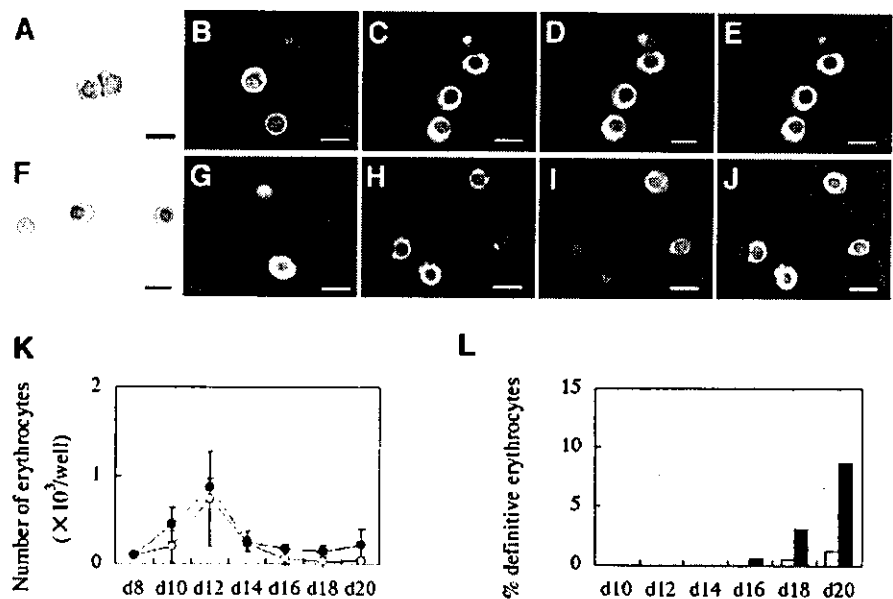
Nestin (718 bp), 5'-GGCAGCGTTGGAACAGAGGTTGG-3' (sense) and 5'-CTAAACTGGAGTGGTCAGGGCTG-3' (antisense);
 AFP (678 bp), 5'-AGAACCTGTACAAAGCTGTG-3' (sense) and 5'-GACAGCAAGCTGAGGATGTC-3' (antisense);
 REX1 (489 bp), 5'-CGCGGTGTGGCCCTTATGTG-3' (sense) and 5'-TCTCAGGGCAGCTCTATTCTC-3' (antisense); and
 β -actin (280 bp), 5'-GCAGGAGATGGCCACGGCGCC-3' (sense) and 5'-TCTCCTTCTGCATCCTGTCCGC-3' (antisense).

The PCR reactions were carried out as follows: ϵ -globin, 35 cycles; γ -globin, 35 cycles; β -globin, 35 cycles; ζ -globin, 35 cycles; α -globin, 35 cycles; EPOR, 40 cycles; Brachyury, 45 cycles; FLK1, 40 cycles; SCL, 45 cycles; LMO2, 40 cycles; MYB, 40 cycles; GATA2, 35 cycles; Nestin, 35 cycles; AFP, 35 cycles; REX1, 35 cycles; β -actin, 35 cycles. The analysis of globin gene expression in individual erythroid colonies was performed for 40 cycles. PCR products were visualized by 1.5% agarose gel electrophoresis using ethidium bromide staining. cDNA from cynomolgus monkey bone marrow or K562 cells was used as a positive control. For semi-quantitative comparisons, samples were normalized by dilution to give equivalent signals for β -actin. DNA sequencing was performed for genes from which we were unable to obtain adequate PCR products from the positive control.

Flow cytometric analysis and cell sorting

The cells induced from CMK6 were harvested sequentially with cell dissociation buffer (Invitrogen), filtered through a 70 μ m nylon cell strainer (Falcon), and incubated in human IgG for 30 minutes to block the non-specific binding of FC γ -receptors. The cells were then incubated with PE-conjugated CD34, or unconjugated FLK1 and KIT antibodies for 30 minutes. Samples staining with unconjugated antibodies were then incubated with APC-conjugated goat anti-mouse antibody (BD Pharmingen) for 30 minutes. The cells were then washed and analyzed using a FACScaliber with the CellQuest program (Becton-Dickinson). Forward- and side-scatter plots were used to exclude MEF or OP9 stromal cells, and propidium iodide co-staining was used to exclude non-viable cells. Mouse IgG1 (Dako) was used as an isotype control. On day 10, the cultured cells were harvested and labeled with CD34 antibody as described above. CD34-positive cells were collected using a FACSVantage flow cytometer

Fig. 1. Development of primitive and definitive erythrocytes. Primate ES cells (4×10^3 cells/well) were cultured onto OP9 stromal cells for 6 days. The induced cells were harvested and re-cultured at a concentration of 1×10^5 cells per well onto fresh OP9 stromal cells, with or without 10 U/ml EPO. The floating cells were harvested every other day and analyzed by May-Giemsa staining and immunostaining against human hemoglobin (Hb), fetal hemoglobin (HbF) and embryonic hemoglobin (HbEmb). (A-E) Day 12; (G-J) day 18. (A,F) May-Giemsa staining of erythrocytes. (B,G) Hb (FITC) and HbF (Cy3) staining of erythrocytes. (C-E,H-J) Hb (FITC) and HbEmb (Cy3) staining of erythrocytes. Merged images are shown in B,E,G and J. Nuclei were labeled with Hoechst 33342 in B-E and G-J. Scale bars: 10 μ m. (K) Sequential analysis of the number of erythrocytes, with (black circles) or without (white circles) EPO. (L) Sequential analysis of the proportion of definitive erythrocytes (EryD) among total erythrocytes, with (black columns) or without (white columns) EPO. EryD were defined as Hb- and HbF-positive, HbEmb-negative erythrocytes, whereas primitive erythrocytes were Hb-, HbF- and HbEmb-positive. Data represent the mean \pm s.d. of triplicate wells. Representative results from one of three independent experiments are shown.



(Becton-Dickinson) and re-plated at a concentration of 2×10^4 cells per well onto fresh OP9 stromal cells in α MEM supplemented with 10% FCS and 50 μ M 2ME, in the presence or absence of EPO (10 U/ml). Floating cells were processed for May-Giemsa staining, immunostaining and RT-PCR analysis.

Results

Primitive and definitive hematopoiesis developed sequentially in primate ES cells co-cultured with OP9 stromal cells

Initially, we tried to induce hematopoietic differentiation from ES cells co-cultured on OP9 stromal cells without any additional cytokines or growth factors in the culture medium. Floating hematopoietic cells, which mainly consisted of large nucleated erythrocytes, first appeared on day 8 of the differentiation induction (Fig. 1A). Immunostaining with antibodies against anti-human hemoglobins demonstrated that all of the large erythrocytes were positive for Hb, HbF and HbEmb (Fig. 1B-E), a pattern that corresponds to primitive erythrocytes (EryP). Thereafter, small nucleated or enucleated erythrocytes appeared faintly on day 16 or day 18 (Fig. 1F). Many of the small erythrocytes were positive for Hb and HbF, but negative for HbEmb (Fig. 1G-J), a pattern that corresponds to definitive (fetal) erythrocytes (EryD). These results suggested that EryP and EryD sequentially developed under the culture conditions.

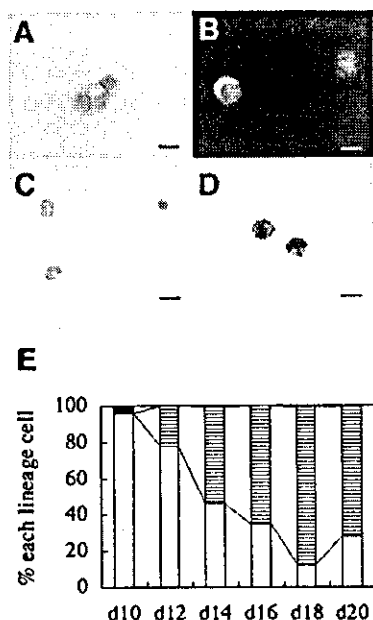


Fig. 2. Development of megakaryocytes and myeloid cells. (A,B) May-Giemsa staining (A, left cell) and CD41 (Cy3) staining (B) of megakaryocytes on day 12. Nuclei were labeled with Hoechst 33342 in B. (C,D) May-Giemsa staining (C) and myeloperoxidase staining (D) of myeloid cells on day 18. Scale bars: 10 μ m. (E) Sequential analysis of the percentages of erythrocytes (white columns), myeloid cells (striped columns) and megakaryocytes (black columns) among total hematopoietic cells in the presence of EPO. Each column represents the mean of triplicate wells. Representative results from one of three independent experiments are shown.

Time-course analysis demonstrated that the number of erythrocytes increased to a maximum on day 12 but gradually decreased thereafter (Fig. 1K). The presence of exogenous EPO resulted in more prominent EryD development (Fig. 1L), although it exerted only minor effects on the number of erythrocytes (Fig. 1K).

CD41-positive megakaryocytes appeared on day 8 (Fig. 2A,B), and MPO-positive myeloid lineage cells appeared on day 12 (Fig. 2C,D). Time-course analysis demonstrated that the number of megakaryocytes peaked on day 10 but decreased thereafter, whereas myeloid lineage cells gradually increased until they comprised more than half of the total number of hematopoietic cells on day 14 and thereafter (Fig. 2E).

The sequential expression of embryonic (ϵ and ζ), fetal (γ) and adult (β and α) globin genes in erythrocytes cultured in the presence of EPO was examined using RT-PCR analysis. As shown in Fig. 3, ϵ and ζ globin genes were expressed on day 6, and their expression was upregulated to high levels on day 8 and thereafter. The expression of the γ and α globin genes remained at low levels on day 8 but was upregulated on day 12 and thereafter, whereas β globin gene expression remained at low levels on day 12 and was not upregulated until day 18. Expression of EPOR, which was detected in undifferentiated ES cells, declined on day 4 but was upregulated again on day 8. These results confirmed that erythropoiesis begins on day 6 and the transition from primitive to definitive erythropoiesis occurs on day 12 at the mRNA level.

Adherent hematopoietic cell clusters developed from primate ES cells

In the OP9 co-culture system, adherent hematopoietic cell clusters first appeared on day 8, irrespective of the presence of

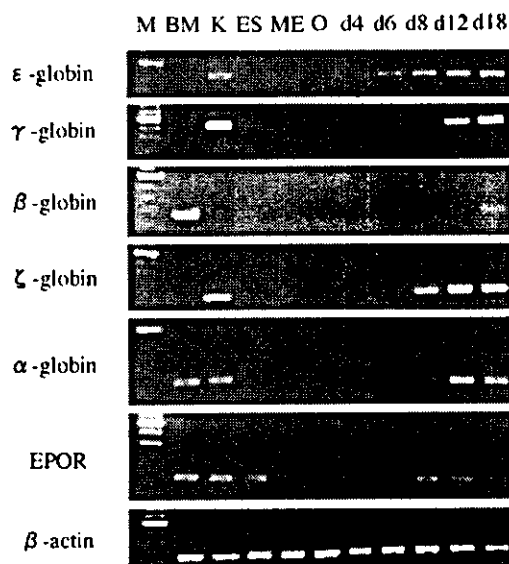


Fig. 3. Sequential RT-PCR analysis of embryonic (ϵ and ζ), fetal (γ) and adult (β and α) globin expression in erythrocytes during liquid culture differentiation. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; ES, undifferentiated ES cells; ME, mouse embryonic fibroblasts; O, OP9 stromal cells; d, days after the induction of differentiation in the presence of EPO.