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Antigen-specific IL-23/17 pathway activation by murine semi-mature DC-like cells

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ABSTRACT

We analyzed the phenotype and function of bone marrow-derived dendritic cells (DCs) induced *in vitro* without using any serum during the late stage of cultivation. These 'serum-free' DCs (SF-DCs) possessed the ability to induce T cell proliferation as well as antibody responses, indicating that they were functional DCs. Surprisingly, the SF-DCs akin to semi-mature DCs in terms of both phenotypic and functional characteristics. The SF-DCs did not produce IL-12 but produced large amounts of IL-23 following lipopolysaccharide stimulation. The antigen-specific production of IL-17 by $CD4^+$ T cells co-cultured with OVA-loaded SF-DCs was significantly higher than that with OVA-loaded conventional DCs. These results suggest that SF-DCs tend to produce IL-23 and can consequently induce the IL-17 producing $CD4^+$ T cells. The semi-mature DC-like cells reported here will be useful vehicles for DC immunization and might contribute to studies on the possible involvement of semi-mature DCs in Th17 cell differentiation.

Introduction

Dendritic cells (DCs) are the most potent antigen (Ag)-presenting cells in the immune system [1]. Inaba et al. and Lutz et al. reported the successful generation of a large number of murine bone marrow-derived DCs (BM-DCs) in the presence of xenologous sera, such as fetal bovine serum (FBS) [2,3]. Establishment of such BM-DC generation methods enabled us to study the functional characteristics of DCs, including the differentiation stages of DCs. However, when DCs are used as vehicles to induce Ag-specific immunity or tolerance, these cells cultured *in vitro* generate unwanted cellular and humoral immune responses against molecules present in FBS [4–6]. Some protocols have described the generation of murine BM-DCs under serum-free conditions using commercially available media [7], however, the recovery of DCs in these studies were too low to analyze the influence of FBS deprivation on the DC functions and the protein component of the media is not open. Hence, it is important to establish a protocol for the induction of murine BM-DCs *in vitro* not involving the use of any serum with predefined components.

Upon encountering Ags and inflammatory cytokines [8], DCs show upregulated expression of the MHC and co-stimulatory molecules. In general, DCs are tolerogenic when immature, and immunogenic when mature. DCs showing moderate expression of co-stimulatory molecules without secretion of adequate pro-inflammatory cytokines produce immune tolerance rather than immune activation [9,10]. DCs of this type are termed 'semi-matured' DCs [11]. Maturation stimuli differ in their ability to induce semi-mature or fully mature DCs, based on their capacity to produce cytokines. Signals that induce cytokine production by DCs and full maturation are associated with microbial recognition as represented by toll-like receptors. After subcutaneous injection of semi-mature DCs, micro-injuries might allow access to endogenous ligands for TLR4, which could induce full maturation of the injected DCs [11].

In this study, we first showed that serum deprivation in the late state of culture, a large number of DCs were recovered that were akin to semi-mature DCs [11]; high expression levels of the DC maturation markers, but were unable to induce the production of IL-12. We then showed that the semi-mature DC-like cells induced the production of large amounts of IL-23 following stimulation with lipopolysaccharide (LPS). In addition, we showed that the $CD4^+$ T cells co-cultured with semi-mature DC-like cells produced a large amount of antigen-specific IL-17 and that splenocytes obtained from mice immunized with the semi-mature DC-like cells loaded with ovalbumin (OVA), re-stimulated with OVA, produced a large amount of IL-17.

Materials and methods

Animals and antibodies. BALB/c mice (Sankyo Lab Service, Tokyo) were used at 6–8 weeks of age. DO11.10 TCR-transgenic mice were kindly provided by Dr. M. Kubo (RIKEN RCAI, Japan). Most of the

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monoclonal Abs used in this study were purchased from BD Pharmingen. FITC- and PE-conjugated streptavidin were purchased from eBiosciences (San Diego).

Media. The conventional DC-generating medium (FBS-RPMI) consisted of RPMI1640 medium supplemented with 10% heat-inactivated FBS. The serum-free HY medium (SF-HY) consisted of IMDM (SIGMA) supplemented with 8 μg/mL of bovine insulin (SIGMA) and 5 μg/mL human transferrin (Boehringer–Mannheim). Both the FBS-RPMI and SF-HY media also contained 10–20 ng/mL of recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) purified (Peprotech) or in culture supernatants from GM-CSF-expressing L1.2 cells cultured in FBS-RPMI or SF-HY.

Generation of bone marrow-derived DCs and determination of cytokines. Conventional bone marrow-derived DCs (FBS-DCs) were generated in FBS-RPMI, as described previously [3]. During the cultivation of FBS-DCs, the non-adherent cells were collected, washed once with ice-cold PBS (−), resuspended with SF-HY, and returned to the culture wells also washed once with PBS (−). After cultivation for two additional days, the DCs (hereafter referred to as SF-DCs) were collected and used for the experiments. FBS-DCs and SF-DCs were harvested on day 10 and stimulated, at a cell density of 5×10^5 cells/mL, with 1.0 μg/mL of LPS (*Escherichia coli*; SIGMA), followed by incubation at 37 °C for 0–96 h. For DC surface marker expression analysis, cells were harvested at 24 h after LPS stimulation. Supernatants were collected at each time point after the stimulation, and the levels of IL-12 p70, TNF-α, and IL-23 were determined using ELISA kits (IL-12 p70; BD Biosciences, TNF-α and IL-23; eBiosciences).

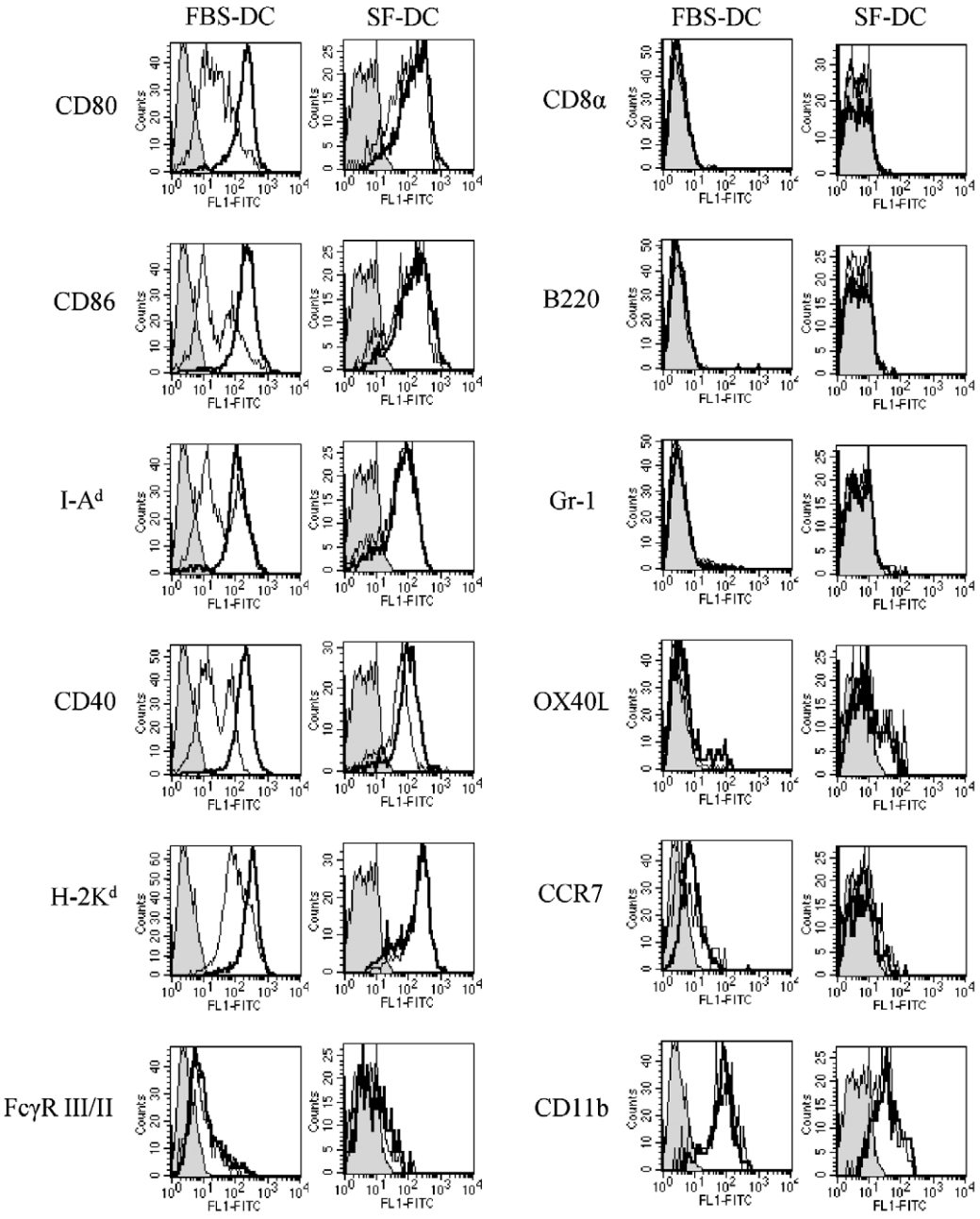

Fig. 1. Serum-deprivation specifically increased the expression of the activation markers on DCs. DCs were harvested at day 11 and stained using a panel of FITC-labeled Abs, PE-labeled anti-CD11c Abs and PI, to analyze the cell-surface markers (left: DC markers; right: cell adhesion molecules). The gray histograms show the expressions on the unstained DCs, and the thin lines show the expressions on DCs stimulated by factors other than LPS. The LPS-stimulated DCs are shown as bold lines.

Flow-cytometric analysis. Monoclonal Abs reactive with the respective surface markers were detected using FACS (BD). All staining steps were performed in PBS with 0.5% FBS, 5 mM EDTA, and 0.1% NaN_3.

In-vitro proliferation assay. The T cell-enriched population (T cells) was isolated from spleen cells prepared from BALB/c mice immunized with ovalbumin (OVA, SIGMA) by a panning method using rabbit IgG specific for mouse IgG (Cappel, Ohio). Antigen was loaded by incubating the FBS-DCs or SF-DCs with 100 μg/mL of OVA or human serum albumin (HSA) at 37 °C for 2.5 h. Increasing amounts of OVA- or HSA-loaded DCs were used to stimulate T cells (3×10^5 cells) derived from mice immunized with 100 μg of OVA one week previously. The proliferative activity of the T cells was determined 2–4 days later on the basis of ^{3}H-thymidine uptake (0.5 μCi/well) during the previous 18 h.

Immunization with DCs and ELISA. Mice were subcutaneously immunized with 1×10^6 DCs that had been harvested on day 10 and then loaded during the LPS-induced maturation period with 100 μg/mL of purified chicken IgY or OVA at a cell density of 5×10^5 cells/mL overnight. Control animals were immunized with DCs that was not loaded with IgY or OVA, but been pulsed with LPS. The purities of both the FBS-DCs and SF-DCs were >90% on days 10 and 11. Mice immunized with OVA-loaded DCs were further boosted by intravenous injection of OVA at 10 μg/mouse at 7 weeks after the DC immunization. One week later, the antibody responses were evaluated by conventional ELISA using IgY or OVA as the antigen. Alkaline phosphatase-conjugated goat anti-mouse IgG Abs (Southern Biotechnology Associates) were used as the second antibodies. The end-point titers were defined as the highest serum dilution producing an OD value 3-fold higher than that of the pre-immune serum.

Antigen-specific IL-17 production. Purified $CD4^+$ T cells were isolated from the spleen of DO11.10 TCR-transgenic mice using $CD4^+$ T cell isolation kit (Miltenyi Biotec). FBS-DC or SF-DC were incubated for 24 h in the presence or absence of 100 μg/mL OVA and stimulated with 1.0 μg/mL LPS for the last 3 h. After extensive washing, the FBS-DC or SF-DC (1×10^5 cells/mL) were co-cultured with OVA-specific $CD4^+$ T cells (1×10^6 cells/mL) for 4 days and the culture supernatants were harvested and store at −30 °C until use.

Mice were immunized with FBS-DCs or SF-DCs and antigen-specific IL-17 production was determined as described previously [12]. Mice were subcutaneously immunized with OVA-loaded FBS-DCs or SF-DCs at the dose of 1×10^6 cells/mouse and booster was administered 1 week after the DC injection. At 2 weeks after the DC immunization, the splenocytes from each of the groups of mice were re-stimulated with 100 μg/mL of OVA or HSA. Supernatants harvested from splenocytes were collected 48 h after the stimulation, and the levels of IL-17 were determined using ELISA kits (eBiosciences).

Real-time RT-PCR. The expression of IL-12 p35, IL-12/23 p40, and IL-23 p19 mRNAs was detected by the SYBR Green-based real-time RT-PCR technique. Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized using SuperscriptII RT (Invitrogen). The sequences of the Real-time RT-PCR primers used in this study were shown in Supplementary Table. Samples were normalized by housekeeping gene *GAPDH*.

Results

SF-DCs express high levels of DC maturation markers, similar to mature DCs

Culturing bone marrow cells in SF-HY for the last 2 days in DC induction schedules provided the highest purity and yield of $CD11c^+$ cells (Supplementary Fig. 1). We analyzed phenotype of

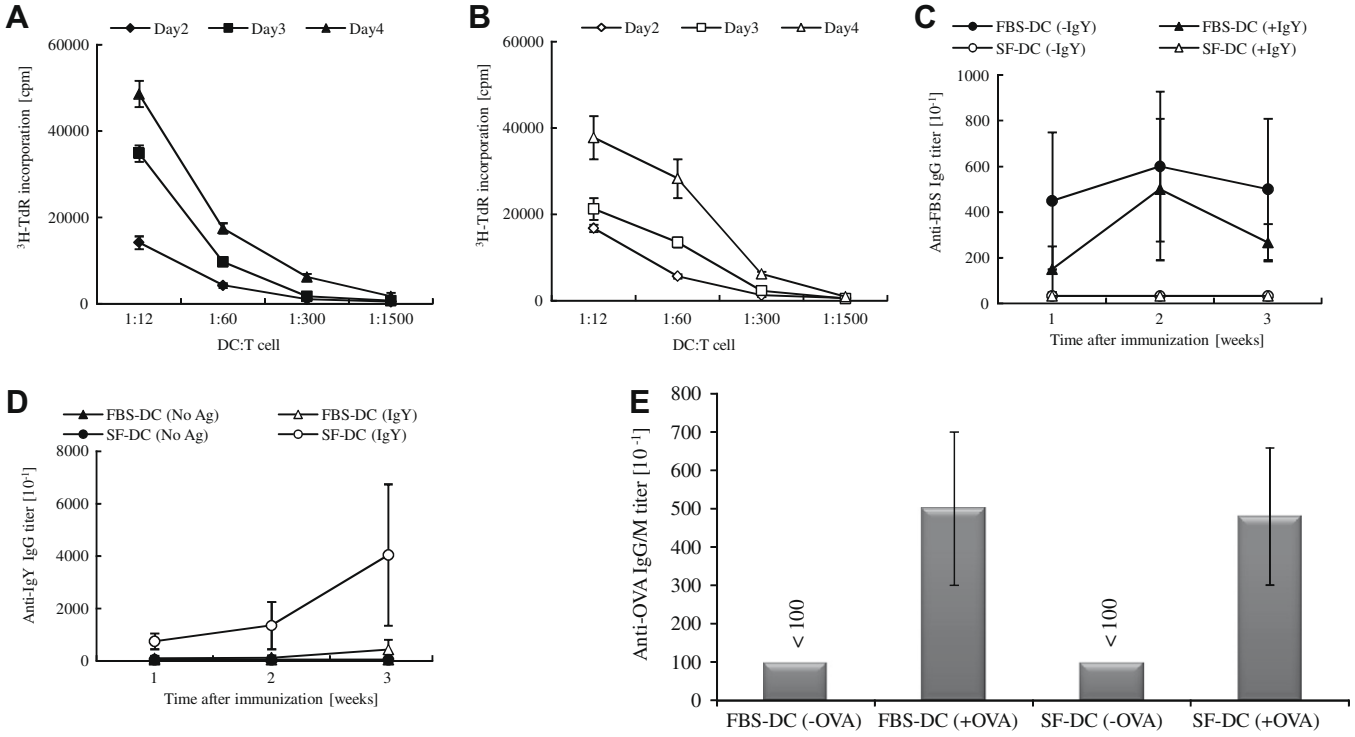

Fig. 2. SF-DCs induced Ag-specific T cell and B cell responses without inducing antibody response against FBS-derived antigens. The proliferation of OVA-specific T cells stimulated by Ag-loaded FBS-DC (A) or SF-DC (B) was determined after 2, 3, and 4 days by examining the incorporation of ^{3}H-thymidine over a period of 18 h. Sera obtained at 1–3 weeks after the immunization with chicken IgY-loaded or unloaded FBS-DCs and SF-DCs were measured by ELISA to determine the titers of anti-FBS IgG antibody (C) and anti-IgY IgG antibody (D). After the OVA-loaded DCs and booster of 10 μg OVA/mouse injections, anti-OVA IgG/M antibody titers were determined by ELISA (E). The average antigen-specific antibody titers (mean ± SD) in each group are shown.

SF-DCs. Even when SF-DCs were not stimulated with LPS, the cells expressed DC maturation markers at much higher levels than that unstimulated FBS-DCs (Fig. 1, left). The expression levels of the maturation markers in the unstimulated SF-DCs were similar to those in the FBS-DCs stimulated with LPS. Because culture of cells in serum-free medium is well known to upregulate the expression of cell adhesion molecules, we also evaluated the expressions of other cell-surface molecules. Surprisingly, no significant differences in the expression levels of other surface molecules were observed between the FBS-DCs and SF-DCs (Fig. 1, right). These results suggest that the increase in the expression levels of the maturation markers on the SF-DCs was not due to non-specific upregulation of cell-surface molecules by serum deprivation. We also examined whether the removal of FBS from the DC generation medium might directly affect the expression of these markers. The expression levels of the maturation markers were upregulated, depending on the FBS concentration (Supplementary Fig. 2). Moreover, enhanced expressions of these cell-surface markers were also dependent on the duration of culture after serum deprivation (Supplementary Fig. 3). These results suggest that SF-DCs generated by serum deprivation during the late stage of the DC generation protocol show a high likelihood of differentiating into DCs expressing high levels of the maturation markers.

SF-DCs possess the ability to induce antigen-specific T cell proliferation as well as an antibody response

Because the SF-DCs expressed high levels of the activation markers, we evaluated the antigen presentation ability of the DCs using enriched OVA-specific T cells. Both the FBS-DCs (Fig. 2A) and the SF-DCs (Fig. 2B) that had been loaded with OVA exhibited OVA-specific T cell proliferation, in a manner dependent on the culture time and the DC: T cell ratio. These results suggest that the SF-DCs, like FBS-DCs, were capable of Ag-specific T cell stimulation.

BALB/c mice immunized with FBS-DCs pulsed or not pulsed with IgY exhibited a strong IgG response against FBS (Fig. 2C). However, no anti-FBS IgG was detected in the mouse sera ($\geqslant$1/100 dilution) throughout the 3 weeks observation period after SF-DCs immunization. Moreover, SF-DCs immunization induced significantly higher IgY-specific antibody titers than FBS-DCs immunization at 1–3 weeks after the immunization (Fig. 2D). Next, we immunized BALB/c mice with DCs that had been pulsed with OVA plus LPS, administered an OVA booster, and then determined the serum Ab titers in a manner similar to that after the IgY-pulsed DC immunizations. The results revealed that SF-DCs could induce OVA-specific Abs as potently as the FBS-DCs (Fig. 2E). Taken together, these data suggest that deprivation of FBS for 3 days during

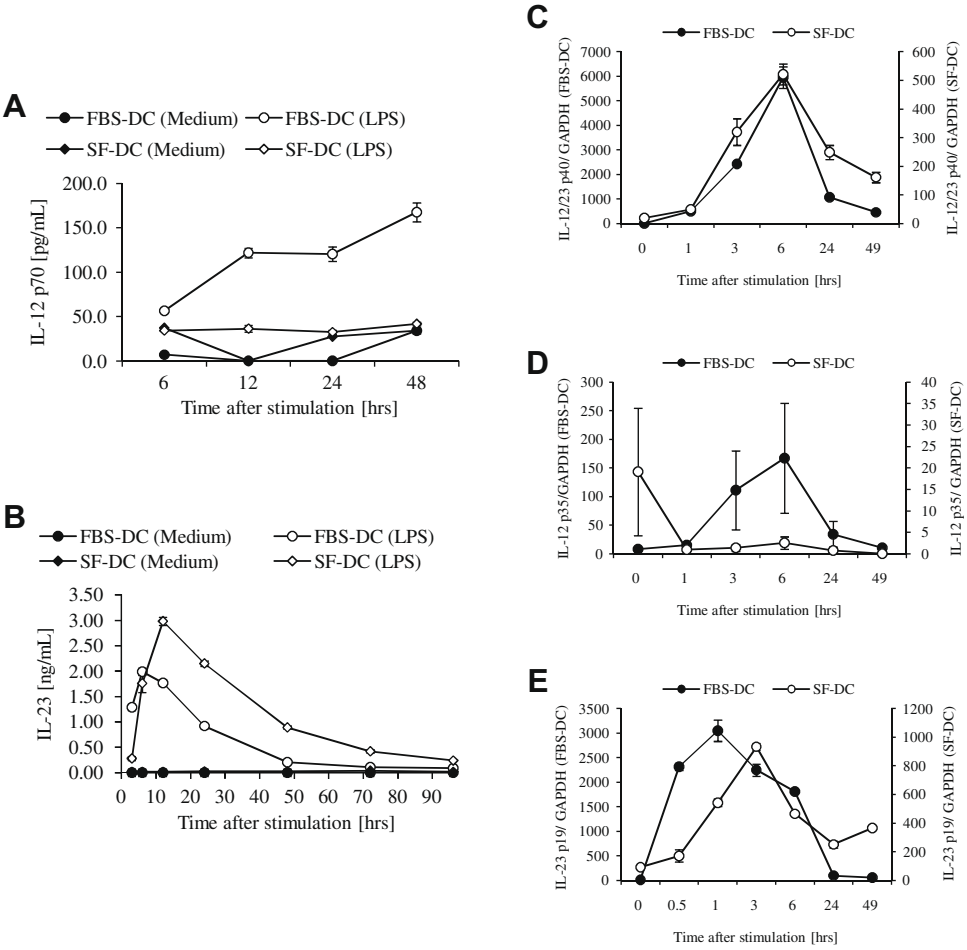

Fig. 3. SF-DCs do not produce IL-12 but produce large amounts of IL-23 after LPS stimulation. (A) FBS-DCs and SF-DCs were stimulated in the presence or absence of LPS for 6, 12, 24, or 48 h and IL-12 in the culture supernatants were determined. (B) FBS-DCs and SF-DCs were stimulated in the presence or absence of LPS for 3, 6, 12, 24, 48, 72, or 96 h and IL-23 in the culture supernatants were determined. (C–E) FBS-DCs and SF-DCs were stimulated in the presence of LPS for 0, 0.5, 1, 3, 6, 24, and 49 h and induction of IL-12/23 p40 (C), IL-12 p35 (D), and IL-23 p19 (E) mRNAs were investigated using real-time RT-PCR analysis.

DC generation resulted in the differentiation of the cells into DCs that do not express FBS-derived antigens, but, nonetheless, possess the ability to evoke an antigen-specific antibody response mediated by helper T cells or memory T cells.

SF-DCs do not produce IL-12 but produce large amounts of IL-23 after LPS stimulation

We examined the production of IL-12, TNF-α, and IL-23 proteins and their mRNAs in the SF-DCs and FBS-DCs after LPS stimulation. Increased secretion of IL-12 p70 protein (IL-12) was observed in the culture supernatant of FBS-DCs, while no increase in the secretion of IL-12 by the SF-DCs was observed (Fig. 3A). Similarly, lower amounts of TNF-α were found in the culture supernatants of the SF-DCs than in those of the FBS-DCs (Supplementary Fig. 4). Since SF-DCs did not secrete the IL-12, we examined the expression by the cells of IL-23, which shares the p40 subunit with IL-12. Although the FBS-DCs produced larger amounts of IL-23 than the SF-DCs at 3 h after LPS stimulation, this dominance was reversed at 12 h (Fig. 3B). Higher and more sustained production of IL-23 by the SF-DCs than by the FBS-DCs was observed by 96 h (Fig. 3B).

Then, we investigated the mRNA expression kinetics of IL-12 p35, IL-12/23 p40, and IL-23 p19 in the FBS-DCs and SF-DCs using real-time RT-PCR. LPS stimulation of FBS-DCs resulted in time-dependent expression of IL-12/23 p40 (Fig. 3C), IL-12 p35 (Fig. 3D), and IL-23 p19 (Fig. 3E) mRNAs. Interestingly, LPS stimulation of SF-DCs resulted in almost no expression of IL-12 p35 mRNA (Fig. 3D) in contrast to the similar induction of IL-12/23 p40 (Fig. 3C) and IL-23 p19 (Fig. 3E) mRNAs to that in FBS-DCs.

These results suggest that the SF-DCs differentiated into semi-mature DC-like cells after serum deprivation and secretion of large amount of IL-23 from the cells might be regulated by expression of p35 subunit of IL-12.

Induction of IL-17 production in $CD4^+$ T cells by SF-DCs

Since the most commonly presumed function of IL-23 is to expand differentiated Th17 cells or maintain IL-17 production [13], we compared the abilities of the FBS-DCs and SF-DCs to induce Th17-producing $CD4^+$ T (Th17) cells *in vitro* by co-culture with $CD4^+$ T cells from DO11.10 mice. As we expected, the SF-DCs induced significantly larger amounts of IL-17 production from OVA-specific $CD4^+$ T cells than FBS-DCs (Fig. 4A). Furthermore, we also obtained the similar result *in vivo*. Splenocytes from BALB/c mouse injected with OVA-loaded SF-DCs enhanced OVA-specific IL-17 production compared with FBS-DCs (Fig. 4B).

Discussion

'semi-matured' DCs have been reported to phenotypically show high expression levels of the DC maturation markers, but to have poor ability to induce the production of pro-inflammatory cytokines, such as IL-12 p70 and TNF-α. Upon s.c. injection, the semi-mature DCs have been shown to differentiate into fully mature DCs capable of inducing immune response [11]. The characteristics of our SF-DCs in this study were nearly identical to those of the semi-mature DCs described above.

SF-DCs induced IgY- or OVA-specific antibody responses *in vivo* (Fig. 2D and E), suggesting that they differentiated into immunogenic fully mature DCs in the living body. It is therefore apparent that the SF-DCs induced by serum deprivation differentiated into semi-mature DC-like cells that could further acquire immunogenic functions *in vivo* after s.c. injection. Generation of 'semi-matured' DCs, may depend on exposure to tumor necrosis factor (TNF)-α [11]. It would be of great interest to investigate the effect of addition of TNF-α into our culture. TNF-α may further stimulate the differentiation of semi-mature DC-like cells. Recent findings indicate that *in-vitro* differentiation of DCs in the presence of prostaglandin E2 alters the IL-12/IL-23 balance and promotes differentiation of Th17 cells [14]. It will be of interest to compare the characteristics of DCs differentiated in the presence of prostaglandin E2 and those of the semi-mature DC-like cells.

Anti-FBS Ab responses were undetectable in the sera of mice immunized with SF-DCs in contrast to the case of the sera of mice immunized with FBS-DCs (Fig. 2C). This finding suggests that removal of FBS from the DC culture medium during the last 3 days of DC generation can reduce the ability of the DCs to present FBS-derived antigens to B cells and generation of unwanted antibody response against molecules present in FBS was overcome with the generation of the SF-DCs.

Interestingly, SF-DCs produced the higher amount of IL-23 protein than FBS-DCs (Fig. 3B). Kinetic analyses of mRNA expression of IL-12 and IL-23 subunits after LPS stimulation revealed that the secretion of large amount of IL-23 from the cells might be regulated by depression of p35 subunit of IL-12 (Fig. 3C–E). A previous report [15] suggested that DCs lacking IL-23 p19 induced the enhanced production of IL-12 and that the IL-12 and IL-23 production may be regulated by physiological competition between p35 and p19 for the p40 subunit. The regulated expression of IL-23 by the depression of p35 subunit of IL-12 described here may support this idea, although precise analysis of the mechanism remains to be conducted.

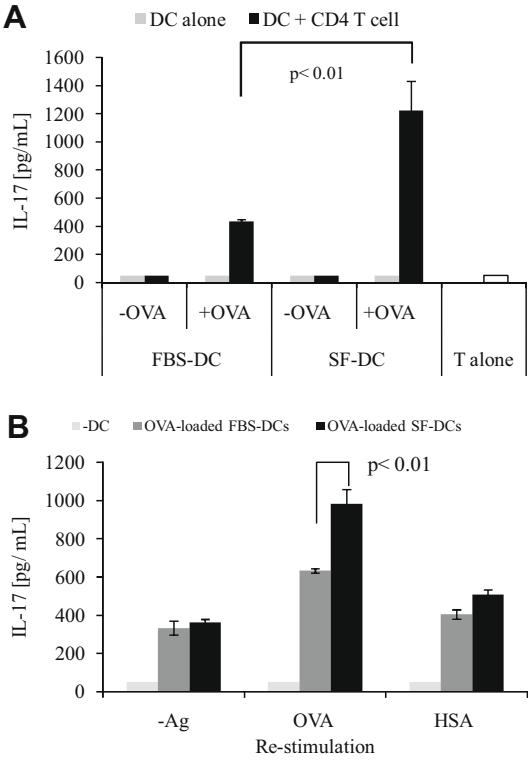

Fig. 4. Antigen-specific induction of IL-17 producing cells by SF-DCs. (A) $CD4^+$ T cells derived from DO11.10 mice were co-cultured with FBS-DCs or SF-DCs loaded with or without OVA for 24 h and stimulated with 1.0 μg/mL of LPS for the last 3 h. The culture supernatants were harvested at 4 days after co-culture, and the productions of IL-17 were analyzed by ELISA. (B) Mice were injected with OVA-loaded DCs, as described in Materials and methods. Pooled splenocytes were isolated from the mice and re-stimulated with 100 μg/mL of OVA for an additional 48 h. The culture supernatants were harvested, and the productions of IL-17 were analyzed by ELISA. $^*P < 0.01$

Today, the most commonly presumed function of IL-23 is to expand differentiated Th17 cells or maintain IL-17 production [13]. In the present study, SF-DCs induced the production of significantly large amounts of IL-23 over the long-term (Fig. 3B). In addition, SF-DCs were more potent at inducing Ag-specific IL-17 production from $CD4^+$ T cells *in vitro* and splenocytes *in vivo* than FBS-DCs (Fig. 4A and B). These results suggest that the SF-DCs tend to produce IL-23 and can consequently induce IL-17 production from the $CD4^+$ T cells. TGF-β and IL-6 are known to be the indispensable factors of Th17 differentiation [13]. Relationship between IL-12/23 pathway activation and expression of TGF-β and IL-6 in Th17 differentiation remains elusive and to be resolved. Th17 cells are thought to be involved in the pathogenesis of various autoimmune diseases [16]. Therefore, SF-DCs might be useful for study on possible involvement of DCs in induction of autoimmune diseases.

Semi-mature DC-like SF-DCs reported here were capable of inducing the antibody response, the secretion of large amounts of IL-23 following LPS stimulation, and of inducing IL-17 production from $CD4^+$ T cells. Semi-mature DC-like SF-DCs are expected to be useful for vehicles for DC immunization and studies on the roles of DCs in the regulation of the IL-23/IL-17 pathway and pathogenesis of autoimmune diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.119.

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